DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

2011 JUN -6 P 2:14

Date: May 9, 2011

From: Fred Hines, Consumer Safety Officer, New Dietary Ingredient Review Team, Division of Dietary Supplement Programs, Office of Nutrition, Labeling and Dietary Supplements, HFS-810

Subject: 75-Day Premarket Notification of New Dietary Ingredients

To: Dockets Management Branch, HFA-305

Subject of the Notification: Creatine nitrate

Firm: ThermoLife International LLC

Date Received by FDA: February 3, 2011

90-Day Date: May 4, 2011

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification and related correspondence for the aforementioned substance should be placed on public display in docket number **FDA-1995-S-0039** as soon possible since the 90-day date is May 4, 2011. Thank you for your assistance.

Fred A Hines

Fred A. Hines, DVM, CSO

FDA-1995-S-0039

RPT 696

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740

APR 19 2011

Ron Kramer
ThermoLife International LLC
3941 East Chandler Boulevard
Phoenix, Arizona, 85048

Dear Mr. Kramer:

This is to inform you that the notification, dated February 3, 2011, which you submitted pursuant to 21 United States Code (U.S.C.) § 350b(a)(2) (section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act (the Act)) was received and filed by the Food and Drug Administration (FDA) on February 3, 2011. Your notification concerned "Creatine Nitrate " which you identify as a new dietary ingredient.

According to your notification, you intend to market the dietary supplement containing "Creatine Nitrate" as a dietary supplement that is intended for ingestion as a capsule and that the proposed maximum serving level will be 3000 mg *per* day (equivalent to 2037 mg creatine and 963 mg nitrate). You also stated that the serving size is 1500 mg, consumed with 8 ounces of water two times *per* day, 30 minutes prior to exercise and within one hour following exercise."

Under 21 U.S.C. 350b(a), the manufacturer or distributor of a dietary supplement containing a new dietary ingredient that has not been present in the food supply as an article used for food in a form in which the food has not been chemically altered must submit to FDA, at least 75 days before the dietary ingredient is introduced or delivered for introduction into interstate commerce, information that is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such new dietary ingredient will reasonably be expected to be safe. FDA reviews this information to determine whether it provides an adequate basis for such a conclusion. Under 21 U.S.C. 350b(a)(2), there must be a history of use or other evidence of safety establishing that the new dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. If this requirement is not met, the dietary supplement is considered to be adulterated under 21 U.S.C. 342(f)(1)(B) because there is inadequate information to provide reasonable assurance that the new dietary ingredient does not present a significant or unreasonable risk of illness or injury.

FDA has carefully considered the information in your submission and the agency has significant concerns about the evidence on which you rely to support your conclusion that a dietary supplement product containing "Creatine Nitrate" will be reasonably expected to be safe under the conditions of use described in your notification.

FDA was unable to establish the identity of your ingredient "Creatine Nitrate" based on the evidence provided in your notification. For example, the structure presented on page 4 is inconsistent with the molecular weight presented on the same page and does not have the charge balance expected of a stabile salt. In addition, you did not provide analytical methods and data to demonstrate the identity and purity of your product. FDA is particularly concerned about the formation of [redacted] likely to be formed under the [redacted] conditions described in the notification. Therefore, it is unclear how the product you intend to market is qualitatively and quantitatively similar to the substances described in the information that you rely on as evidence of safety, or how that information forms the basis for a reasonable expectation of safety under the intended conditions of use.

In addition, FDA was unable to establish the safety of your "Creatine Nitrate" based on the proposed serving levels of "963 mg nitrate per day." For example, you cite "[t]he current Acceptable Daily Intake (ADI) for nitrate [is] 3.7 mg/kg bw/day (equivalent to 222 mg/day for a 60-kg adult)" in Europe on page 17 and in Japan on page 35 and do not explain the basis of the safety of your higher serving levels. Because the identity of your ingredient is not clear FDA did not attempt to evaluate the safety of the component of your ingredients related to creatine.

For the reasons discussed above, the information in your notification does not provide an adequate basis to conclude that the dietary supplement containing "Creatine Nitrate", when used under the conditions recommended or suggested in the labeling of your product, will reasonably be expected to be safe. Therefore, your product may be adulterated under 21 U.S.C. 342(f)(1)(B) as a dietary supplement that contains a new dietary ingredient for which there is inadequate information to provide reasonable assurance that such ingredient does not present a significant or unreasonable risk of illness or injury. Introduction of such a product into interstate commerce is prohibited under 21 U.S.C. 331(a) and (v).

Your notification will be kept confidential for 90 days after the filing date of February 3, 2011. After the 90-day date, the notification will be placed on public display at FDA's Division of Docket Management in docket number FDA-1995-S-0039 (formerly docket number 95S-0316) as new dietary ingredient notification report number 696. Prior to that date, you may wish to identify in writing specifically what information you believe is trade secret or confidential commercial information and an explanation of the basis for this belief.

If you have any questions concerning this matter please contact Dr. Fred Hines, Consumer Safety Officer, New Dietary Ingredients Review Team, at (301) 436-1756.

Sincerely yours,

Dan D. Levy, Ph.D.
Microbiologist, Supervisor
New Dietary Ingredient Review Team
Division of Dietary Supplement Programs
Office of Nutrition, Labeling and Dietary Supplements
Center for Food Safety and Applied Nutrition

NEW DIETARY INGREDIENT NOTIFICATION FOR CREATINE NITRATE

January 6, 2011

RECEIVED
FEB - 3 2011

NEW DIETARY INGREDIENT NOTIFICATION FOR CREATINE NITRATE

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1. DISTRIBUTOR INFORMATION

ThermoLife International
3941 East Chandler Boulevard, Suite 106-212
Phoenix, Arizona 85048

1/19/2011
Date

2. INTRODUCTION

Creatine nitrate (CreN) is a white crystalline powder formed by the ionic bonding of creatine (a nitrogenous organic acid produced endogenously in the liver, pancreas, and kidneys and consumed from high-protein dietary sources such as meat and fish) and nitrate, a common constituent in vegetables and recognized precursor of nitric oxide. As a nitrate salt, CreN disassociates rapidly upon ingestion, releasing creatine which is necessary for the production of energy within muscle tissues, especially under short term high stress situations such as high-intensity exercise, and nitrate, precursor to nitric oxide, the major vasodilator in the body.

On a daily basis, 1-2 grams of creatine are generated *in vivo* with an additional 1-2 grams coming from dietary sources (Pecci and Lombardo, 2000, Shephard *et al.*, 1987). Although endogenous creatine is produced in the liver, pancreas, and kidneys, 95% of creatine within the body is found in the skeletal muscle, where it is utilized in the production of cellular energy (Abelson, 2005). Upon ingestion, creatine from dietary sources is actively transported from the gastrointestinal tract into the blood and distributed throughout the body along with endogenously produced creatine. In comparison to creatine, only small amounts of nitrate are produced *in vivo* while a majority of nitrate is obtained from the diet (estimated to be 40-100 mg/day in the US (Hord *et al.*, 2009). Dietary nitrate is actively transported from the gastrointestinal tract into one of several complex metabolic pathways having numerous feedback loops that optimize availability and limit unnecessary excretion or loss. *In vivo*, nitrate is generated in a localized manner throughout the body with any excess circulating alongside diet-sourced nitrate as a pool of readily available material for the as-needed generation of nitric oxide.

A New Dietary Ingredient Notification for CreN is sought for the use of CreN as a dietary supplement in the United States. The product is intended to be sold in capsule form at a suggested daily intake of 3000 mg/day (equivalent to 2037 mg creatine and 963 mg nitrate *per* day), as an additional source of creatine and nitrogen in the diet. The suggested serving size is 1500 mg, consumed with 8 ounces of water two times *per* day, 30 minutes prior to exercise and within one hour following exercise. Creatine nitrate supplements the diet as an additional source of both creatine and nitrate.

3. DESCRIPTION

Creatine nitrate is a white crystalline powder manufactured from food-grade creatine monohydrate reacted with nitric acid and ethanol. Ionic bonding of nitrate to creatine prevents the association of water found in the common creatine monohydrate formulations. Ionic bonding also allows for rapid dissociation of the substance into creatine and nitrate after ingestion. The general description and chemical structure of CreN is given in Table 1 and Figure 1, respectively.

Table 1. General description of creatine nitrate

Appearance	White crystalline powder
Molecular weight	175.13
Packaging	Polymer drums (25 kg/drum)
Storage	0-30 °C
Stability	One year
Labeling	Creatine nitrate
Functionality in	Dietary supplement

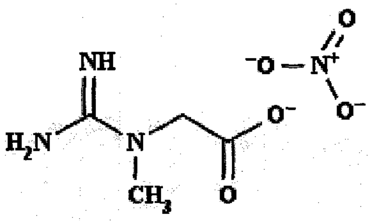

Figure 1. Structure of creatine nitrate

To evaluate dissolution (and indicate potential bioavailability), the dissolution profiles for commercially available CreN, creatine monohydrate (CreM) and buffered creatine (BCre) were determined at room temperature and 37 °C in USP dissolution media adjusted to pH 2.5[1] and 7.4[2] (Pandit *et al.*). Dissolution of a 0.5-gram disk[3] of each substance was conducted in a USP type II dissolution apparatus containing 140 ml of media with a paddle speed of 50 rpm. Dissolution was monitored over time (three hours for CreN and seven hours for CreM and BCre) using (1) a validated high-performance liquid chromatography (HPLC) method to assay creatine concentration in the media and (2) the fact that the dissolution rate constants are directly proportional to the slope (k' in mg/cm^2 h) of the amount of dissolved creatine (mg)/surface area (cm^2) plotted against time. Based on the experimentally determined k' values (Table 2), the researchers concluded that irrespective of temperature and medium pH, CreN had a higher intrinsic dissolution rate constant compared to CreM and BCre. These results indicate that CreN will dissolve more rapidly in the gastrointestinal tract than the commercially available CreM and BCre formulations.

Table 2. Average slope (k' in $mg/\ cm^2 hr$) values of dissolved creatine (mg)/surface area (cm^2) *vs.* time for three commercially available creatine products at room temperature and 37 °C into media buffered to pH 2.5 and 7.4 (*n* = 3 for all temperature-pH combinations).

Test Material	**Room Temperature**		**37 °C**	
	pH 2.5	**pH 7.4**	**pH 2.5**	**pH 7.4**
Creatine nitrate	293.0 ±	278.9±14.	327.3 ±	319.5±14.
Creatine	59.1 ± 2.06	52.1±0.22	97.9 ± 0.88	86.0±5.05
Buffered creatine	71.1 ± 0.59	50.6±0.95	117.9 ±	96.9±9.28

3.1. Manufacturing Process

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[1] A 0.2 *M* potassium biphthalate (KHP) solution was prepared by dissolving 10.21 g of KHP in 250 ml water, adding 211 ml of 0.2 *M* HCl and taking the solution to a final volume of 1000 ml in water.

[2] A 0.2 *M* monobasic potassium phosphate (KH_2PO_4) solution was prepared by dissolving 6.81 g of KH_2PO_4 in 250 ml water, adding 195.5 ml of 0.2 *M* NaOH and taking the solution to a final volume of 1000 ml in water.

[3] Disks, formed by compression in a Carver press at 2000 psi for ten seconds, had a surface area of 1.21 cm^2.

PAGE 6

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3.3. Stability

Creatine nitrate is stable for one year from the date of manufacture when stored in tight containers under a cool (0-30 °C), dry environment (< 75% relative humidity), away from direct sunlight (Table 4) (Thermo-Life International, 2010).

[The remainder of this page is blank]

Table 4. Stability of creatine nitrate over time

	Creatine Nitrate content (% by weight)	Loss on drying (% by weight)	Comments
Batch No. 90910			
0 months	97.5	0.5	
3 months	97.5	0.5	
6 months	97.3	0.5	
9 months	97.3	0.6	
12 months	97.3	0.6	Conforms
Batch No. 91011			
0 months	97.4	0.1	
3 months	97.4	0.1	
6 months	97.3	0.15	
9 months	97.3	0.16	
12 months	97.1	0.18	Conforms
Batch No. 91101			
0 months	97.2	0.4	
3 months	97.2	0.3	
6 months	97.2	0.3	
9 months	97.1	0.3	Conforms
12 months	In progress	In progress	In progress

4. CONDITIONS OF USE

Creatine nitrate is a dietary ingredient which will be used in supplements to provide an added source of creatine and nitrate (recognized precursor to nitric oxide) to the diet. The maximum amount that will be recommended is 3000 mg of CreN *per* day (equivalent to 2037 mg creatine and 963 mg nitrate *per* day). The suggested serving size is 1500 mg, consumed with 8 ounces of water two times *per* day, 30 minutes prior to exercise and within one hour following exercise.

5. HISTORY OF USE

Creatine and nitrate are both produced endogenously by humans and are independently present in natural food sources, with creatine primarily found in meat and fish and nitrate in vegetables and fruits (*e.g.* spinach, lettuce, beets and pomegranates).

Creatine is a naturally occurring substance, produced endogenously by humans and consumed through the diet (Guidi *et al.*, 2008). Although generated in the pancreas, liver and kidney, about 95% of the creatine in the human body is present in muscle tissue (Vikse and

Joner, 1993). In rich dietary sources such as raw bovine meat, heart and tongue, concentrations of creatine plus creatinine (the predominate creatine metabolite) were measured to be 2.49-4.33 mg/g wet tissue with concentrations in liver and kidney being only about 0.26 mg/g wet tissue (Reutersward *et al.*, 1987a, Reutersward *et al.*, 1987b). In fish, fillets of skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Neothunnus macropterus*), milkfish, (*Chanos chanos*) and sole (*Microstomus pacificus*) contained 2.51 mg creatine/g wet weight tissue for sole up to 6.45 mg creatine/g wet weight for yellowfin tuna (Marsh *et al.*, 1990). On a daily basis, 1-2 grams of creatine are generated *in vivo* with an additional 1-2 grams coming from dietary sources (Pecci and Lombardo, 2000, Shephard *et al.*, 1987). Commercially available as a dietary supplement, creatine, primarily in the form creatine monohydrate (CreM), is used as an ergogenic aid to improve exercise performance and to increase fat free muscle (Guidi *et al.*, 2008, Fernandez and Hosey, 2009). One of the first reported uses of creatine supplementation by athletes was during the 1970s when athletes of the Soviet Union competing in the Olympic Games used CreM for its ergogenic potential (Kalinski, 2003). Widespread use of creatine in the US as an athletic aid began in the 1990s (Pecci and Lombardo, 2000). Currently adolescents, in addition to adults, are known to take creatine as a performance-enhancing substance and/or to improve their appearance (Holland-Hall, 2007); in 2003, about 5.6% of high school and 28% of college athletes admitted use of creatine (Chyka, 2003). Creatine is also intentionally consumed for its antioxidant properties (Guidi *et al.*, 2008). Among active duty Air Force personnel, creatine and nitric oxide (consumed form not specified) are among the non-vitamin, non-mineral supplements most commonly reported as being frequently or regularly used (Thomasos, 2007).

Critical for human health, creatine deficiency, when chronic, can be devastating with outcomes including mental retardation, developmental delays and profound depletion of central nervous system creatine and CreP which allows for the development of dystonia and seizures (Galbraith *et al.*, 2006, Schulze, 2003). Although symptoms of the three main creatine deficiency syndromes in humans (*i.e.*, GAMT[5] deficiency, AGAT[6] deficiency and creatine transporter deficiency), which typically onset in the very young although diagnosis is often delayed due to the rarity of these defects and to the similarity of initial symptoms to other more common disorders[7]. Once recognized, oral creatine supplementation can offset partial effects in patients having GAMT and AGAT, but not in patients having creatine transporter deficiency (Galbraith *et al.*, 2006).

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[5] Guanidinoacetate methyltransferase deficiency

[6] Arginine:glycine amidinotransferas deficiency

In other medical applications, dietary supplements containing creatine are gaining in use as nutritional support during the conventional treatment of certain diseases. Creatine is among the amino acid supplements administered as nutritional interventions to patients having Duchenne muscular dystrophy[8] to assist in the preservation of skeletal muscle from the wasting effects of the disease (Radley *et al.*, 2007). Creatine supplementation is also under current consideration for patients with Huntington's disease[9] as a means of preserving cellular energy and counter-balancing the mitochondrial dysfunction that reduces cellular energy as the disease progresses (Ryu *et al.*, 2005) and for patients with glaucoma as a means of slowing down retinal ganglion cell death (Osborne, 2008). In addition to being an antioxidant, creatine is appealing for use in glaucoma patients because administration is simple: the substance is known to reach the retina after oral consumption (Osborne, 2008).

Nitrate, also a naturally occurring substance, is consumed through the diet and to a limited extent is endogenously produced by the arginine-NOS[10] pathway in humans (Lundberg *et al.*, 2004, Lundberg *et al.*, 2008). Necessary for the generation of nitrite and nitric oxide, both of which substances are required for the normal functioning of human vasculature (Hord *et al.*, 2009), about 80% of the nitrate consumed daily in the Western diet is supplied through vegetable matter (Bailey *et al.*, 2009) with secondary dietary sources including drinking water (up to 50 mg/l permitted in Europe and 10 mg/l permitted in the US (Santamaria, 2006)), cured meats 0.009-0.055 mg/g (Hord *et al.*, 2009) and fruit 0.003-0.045 mg/g (Hord *et al.*, 2009). Vegetables vary widely in nitrate content by species – asparagus, garlic and eggplant generally contain nitrate concentrations < 0.20 mg/g fresh matter while spinach, celery and red beet root typically contain concentrations in excess of 2.50 mg/g fresh matter (Santamaria, 2006). Individual product concentrations may also vary (*e.g.* 0.25-0.42 mg/g in eggplant and 0.24-3.87 mg/g in spinach) due to factors related to genotype and soil, light, fertilization, storage, transport, and processing conditions. In human terms, this inherent variability means that nitrate consumption in individuals following DASH[11] diet recommendations could vary as widely as 174 mg/day to 1222 mg/day (Hord *et al.*, 2009), depending on the vegetables and fruits selected (Hord *et al.*, 2009). In general, dietary intakes of nitrate from food are estimated to range from 40-100 mg/day (0.67-1.67 mg/kg bw/day) in the US and 31-185 mg/day (0.52-3.08 mg/kg bw/day) in Europe (Hord *et al.*, 2009).

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[7] Due to a similarity to progressive myoclonic epilepsy, GAMT deficiency may not be accurately diagnosed until the patient is 14 years or so of age, although symptom onset generally occurs from infancy to three years of age.

[8] A lethal X-linked disease caused by a defect in dystrophin, a muscle membrane protein. The disease is characterized by extensive muscle wasting and typically results in death due to respiratory failure by the early 20s (Radley *et al.*, 2007).

[9] A fatal neurodegenerative genetic disorder caused by the expansion of the trinucleotide repeat CAG in the gene coding for the Huntington protein. The disorder is characterized by progressive choreiform movements, psychiatric symptoms and cognitive decline. Symptoms generally become apparent during middle age. There is no cure; progress is relentless and generally causes death within twenty years of diagnosis (Ryu *et al.*, 2005).

[10] Nitric oxide synthase.

Commercially, a major use of nitrate salts (*e.g.*, ammonium nitrate, calcium ammonium nitrate, potassium nitrate, sodium nitrate) is in fertilizers for agricultural applications. Nitrates are also used in the production of nitrate-based explosives (*e.g.*, trimethylolthane trinitrate-nitrocellulose and urea nitrate). Silver nitrate is utilized in mirrors, inks, hair dyes, and silver plating. Sodium and potassium nitrate salts are used as curing agents and food preservatives. Medically, organic nitrates (*e.g.*, glyceryl trinitrate, isosorbide dinitrate and isosorbide mononitrate) are substances primarily prescribed to prevent and relieve angina (Kosmicki, 2009).

6. REGULATORY STATUS

Creatine nitrate is not regulated by FDA and, further, holds no status by EFSA[12]. Alone, creatine is not regulated by the FDA or mentioned in 21 CFR, except as the analyte of primary interest in creatine test systems [§862.1210] for clinical chemistry. Creatine and creatinine are monitored in blood and urine as an evaluative measure of kidney function; concentrations increase with kidney failure and uremia. As a dietary supplement under the conditions of the DSHEA[13], CreM can be purchased in health food stores and pharmacies (IDACE, 2003).

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[11] The Dietary Approaches to Stop Hypertension (DASH) diet recommends a diet rich in vegetables (8-10 servings) and low-fat dairy as a means of reducing blood pressure.

[12] European Food Safety Authority

[13] Dietary Supplement Health and Education Act of 1994

In Europe, the SCF[14] report to the Commission of European Communities (CEC, 2004) recognized that scientific data is available to support an ergogenic effect for creatine and, separately, that creatine is effective at increasing total muscle creatine and improving short term high intensity exercise performance. Regarding safety, the committee recommended against high loading. Although it is noted in the report that interested parties had requested a maximum permitted level of 5 grams of creatine *per* day for regular supplementation with higher allowed levels of use during periods of loading, the SCF recommended intakes of "not more than 3 g creatine *per* day" (equivalent to 50 mg/kg bw/day for a 60-kg adult) (CEC, 2004).

When the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food was asked to render a scientific opinion on the use of CreM as a nutrient source in "foods for particular nutritional uses" (EFSA, 2004), the Panel concluded that the safety of CreM was considered to be similar to that of creatine, that "No specific assessment of exposure of CreM was considered necessary provided that the intake of creatine is within the amounts judged unlikely to pose any risk (2-3 g/day)." In summary, the Panel determination coincided with other SCF findings for creatine (CEC, 2004, SCF, 2000, SCF, 2001) that "consumption of doses up to 3 g/day of supplemental creatine, similar to the daily turnover rate of creatine, is unlikely to pose any risk."

The various nitrate salts are regulated by FDA as food additives, secondary direct food additives, indirect food additives, prior-sanctioned food ingredients (*i.e.*, potassium nitrate and sodium nitrate), direct food substances affirmed as generally recognized as safe (GRAS) (*i.e.*, thiamine mononitrate) and as the active ingredients offered in certain over-the-counter (OTC) drug products for specific uses (Table 5). The current Acceptable Daily Intake (ADI) for nitrate (as the nitrate ion) supported by the Joint FAO/WHO Expert Committee on Food Additives is 0-3.7 mg/kg bw/day.[15]

The current NPDWR (National Primary Drinking Water Regulation) for nitrate published by USEPA on January 30, 1991 (56 FR 3526 (EPA, 1991) (40 CFR 141.62) established a Maximum Contaminant Level (MCL) of 10.0 mg/l, and the requirement that all public water systems must monitor for nitrate at each entry point to the distribution system

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[14] Scientific Committee on Foods

[15] <http://apps.who.int/ipsc/database/evaluations/chemical.aspx?chemID=709>; site accessed December 20, 2010.

(EPA, 2009). As currently applied, the MCL for nitrate is 10 mg/l in water from public or community-sourced drinking water systems, although nitrate levels in water from noncommunity water systems (NCWSs) are allowed to exceed the MCL (up to 20 mg/l) as permitted by the primacy agency (EPA, 2010).

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Table 5. FDA regulatory status of nitrate species

Nitrate Species	Permitted Functionality	Use Limits	Other Considerations	21 CFR Reference
Nitrate	Inorganic component in bottled water	Up to 10 mg/L	None	§165.110
Nitrate	Food additive when combined in curing premixes	NR	Provided that a food additive petition to establish safety has been submitted pursuant to §171.1 and §171.100; petition must include data demonstrating that nitrosamines are not formed in curing premixes containing such food additives	§170.60
Potassium nitrate	Curing agent in the processing of cod roe	Up to 200 ppm in finished roe	None	§172.160
Sodium nitrate	Preservative or color fixative in smoked and cured sablefish, salmon and shad and in meat-curing preparations for the home curing of meat and meat products (including poultry and wild game)	Up to 500 ppm in the finished product	None	§172.170
Sodium nitrate	Secondary direct food additive permitted in food for human consumption: Boiler water additive	NR	None	§173.310
Nitrate salts (including calcium, chromium, fumaratochromium (III), magnesium, sodium and zinc nitrates)	Indirect food additives: Adhesives; Magnesium nitrate as a component in the 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one mixture used as an antimicrobial agent in polymer latex emulsions	NR except as described for magnesium nitrate	Limited to usage as adhesive components; Magnesium nitrate concentration is equivalent to the isothiazolone active ingredients	§175.105
Magnesium nitrate	Indirect food additives: Resinous and polymeric coatings for	As described for magnesium nitrate	Magnesium nitrate concentration is equivalent to the isothiazolone active	§175.320

Nitrate Species	Permitted Functionality	Use Limits	Other Considerations	21 CFR Reference
	polyolefin films; Magnesium nitrate as a component in the 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one mixture used as an antimicrobial agent in in emulsion-based silicone coatings		ingredients	
Ammonium nitrate	Indirect food additive: component in paper and paperboard in contact with dry food	Not to exceed the amount required to accomplish the intended physical or technical effect	None	§176.180
Silver nitrate and cupric nitrate salts	Indirect food additives: Slimicides as antimicrobial agents in paper and paperboard	Not exceed the amount necessary to accomplish the intended technical effect	None	§176.300
Sodium nitrate-urea complex	Indirect food additives: Plasticizer in glassine and greaseproof paper and paperboard	Not to exceed the amount required to accomplish its intended technical effect or exceed 15 percent by weight of the finished paper	None	§176.320
Sodium nitrate and potassium nitrate	Specific prior-sanctioned food ingredients	As described	Use subject to prior sanctions issued by the U.S. Department of Agriculture for use as sources of nitrite, with or without sodium or potassium nitrite, in the production of cured red meat products and cured poultry products	§181.33
Thiamine mononitrate	Direct food substances affirmed as generally	cGMP	None	§184.1878

Nitrate Species	Permitted Functionality	Use Limits	Other Considerations	21 CFR Reference
	recognized as safe			
Silver nitrate, thiamine mononitrate (vitamin B1 mononitrate), potassium and sodium nitrate, and phenylmercuric nitrate	Drug products containing certain active ingredients offered over-the-counter (OTC) for certain uses: Astringent drug product, weight control drug products, orally administered menstrual drug products, topical antimicrobial drug products and vaginal contraceptive drug products	NR	None	§310.545

cGMP = current good manufacturing practice; NR = not reported;

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In Europe, nitrate is an approved food additive. The current Acceptable Daily Intake (ADI) for nitrate, 3.7 mg/kg bw/day (equivalent to 222 mg/day for a 60-kg adult), was established by the SCF and was confirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002 (EFSA, 2008). At the time, JECFA calculated oral nitrate exposures from other sources (*e.g.*, drinking water and cured meat) to average 35-44 mg/person/day (0.58-0.73 mg/kg bw/day). The current maximum level of nitrate allowed by Council Directive 98/83/EC in surface water used as drinking water is 50 mg/l (EFSA, 2008).

7. ABSORPTION, DISTRIBUTION, METABOLISM AND ELIMINATION (ADME)

Although produced in the liver, pancreas, and kidneys from the essential amino acids glycine, *L*-arginine and *L*-methionine, about 95% of creatine within the body is found within the skeletal muscle (Abelson, 2005) where it is utilized in the production of cellular energy. Dietary creatine, primarily from meat, fish and poultry, is transported rapidly from the gastrointestinal tract into the liver and kidneys where it is dispersed throughout the body along with endogenously produced creatine. On a daily basis, 1-2 grams of creatine are generated *in vivo* with an additional 1-2 grams coming from dietary sources (Pecci and Lombardo, 2000, Shephard *et al.*, 1987).

Functionally, creatine plays a critical role in maintaining energy homeostasis in all cells in vertebrate organisms, but especially those cells with intermittent high energy requirements (Ide *et al.*, 2010, McLeish and Kenyon, 2005). In a reversible reaction with ATP (adenosine triphosphate), 60-67% of available creatine is catalyzed by creatine kinase to form phosphocreatine (PCre) and ADP (adenosine diphosphate). ADP is then provided a "high energy phosphoryl" on demand from PCre, a short-term energy storage form or buffer, to resynthesize ATP, the primary energy source within a cell (McLeish and Kenyon, 2005). The availability of phosphocreatine is the rate-limiting step in the reversible conversion of ADP and ATP (Pecci and Lombardo, 2000). During high demand periods (such as high intensity exercise) stores of ATP are depleted rapidly, usually in fewer than 20 seconds (Abelson, 2005). With sufficient supplementation the available creatine in human skeletal muscle increases (Moghadam *et al.*, 2008) from the normal range around 3.9 g/kg to the saturation limit of 6.5-7 g/kg (Pecci and Lombardo, 2000). Phosphocreatine, which is usually present in muscle at twice the molar concentration of creatine, increases with supplementation by 12-18% (Juhn and Tarnopolsky, 1998). Normal to saturated concentrations of total creatine in muscle range from about 90 mmol/kg to up to 160 mmol/kg (16.4 g/kg to up to 29.3 g/kg; (Pecci and Lombardo, 2000)). In addition to being critical for general energy production, creatine is also essential for brain function (Wu *et al.*, 2009)and has been shown to exert anti-oxidative activities, reduce inflammatory responses, support mitochondrial genome stability, and improve glucose tolerance in humans (Guidi *et al.*, 2008, Wu *et al.*, 2009, Bassit *et al.*, 2008, Gualano *et al.*, 2010, Nomura *et al.*, 2003)

Under conditions of normal dietary consumption, about half of all creatine synthesized or consumed (~2 g/day) is eliminated *via* renal excretion, primarily as creatinine with small amounts of creatine (Abelson, 2005, Moghadam *et al.*, 2008, Wu *et al.*, 2009). Creatinine, the major metabolite of creatine is generated at a fairly constant rate *via* nonenzymatic production and the irreversible loss of a water molecule. Sarcosine, a minor metabolite, is also excreted in the urine along with methylamine and formaldehyde, its degradation products and known cytotoxic agents. Under conditions of excess supplementation (> 20 g *per* day), concentrations of creatinine in serum may increase to a limited extent (*e.g.*, from a normal upper limit of 1.5 mg/dl serum creatinine to 2.3 mg/dl (Francaux and Poortmans, 2006), while the relative amount of unconverted creatine excreted in the urine tends to increase markedly. Both creatine and creatinine effects are reversible with discontinuation of supplementation (PDR, 2001). Reduction of serum creatinine to pre-loading levels can take as long as six weeks (Taes and De Vriese, 2005) as "fully loaded" muscles can retain elevated stores of creatine for up to five weeks without additional supplementation (Shao and Hathcock, 2006).

Depending on creatine load, the quantities of methylamine and formaldehyde excreted also increase, from an average of 0.69 ± 0.06 mg and 64.78 ± 16.28 μg, respectively, in the 24-hour urines of 20 healthy young men prior to supplementation to 6.41 ±1.45 mg and 290.4 ± 66.3 μg after a 14-day supplemental regimen of 21 g creatine/day (Poortmans *et al.*, 2005). Even with excessive creatine loading, urinary concentrations of sarcosine and, *N*-nitrososarcosine a carcinogenic nitrosation product, did not increase. Although elevated, urinary methylamine and formate (the rapidly produced end-product of formaldehyde) concentrations also remained at levels within the upper normal ranges for healthy humans, up to 35 mg/day and 20 mg/day, respectively (Francaux and Poortmans, 2006).

The formation of *N*-nitrososarcosine, a known carcinogen, from ingested creatine after exposure to the high acidity and high nitrite levels found in the stomach has periodically been voiced as a concern (Archer, 1999, Archer, 2004a, Archer, 2004b). In a double-blind placebo controlled study (Derave *et al.*, 2006), excessive creatine loading (>20 g/day) was shown to not increase urinary *N*-nitrososarcosine concentrations in healthy young men and women. In the study, volunteers (*n* = 12 men, *n* = 4 women, 18.8 ± 0.3 years of age) were randomly assigned to the maltodextrin placebo or CreM test group. For the first week, 20 grams total of the placebo or supplement were consumed in four evenly divided doses each day. For the next 19 weeks, one 5 gram dose was taken daily. Twenty-four hour urine samples were collected at baseline, after the first week and at the end of the study. Mean urinary *N*-nitrososarcosine concentrations in the placebo and dose groups did not vary significantly over the 20-week supplementation study: Concentrations in the placebo and dose groups at baseline were 1416 ± 464 and 1456 ± 363 pmole/24 hour, respectively, and 1958 ± 227 and 2023 ± 1853 pmole/24 hour at Week 1 and 4018 ± 7347 and 1571 ± 5421 at Week 20. The authors concluded that creatine ingestion did not increase urinary levels of *N*-nitrososarcosine.

Like creatine, nitric oxide (NO), the end-product of nitrate metabolism, is critical for skeletal muscle and brain function (Wu *et al.*, 2009). The major functions of accumulated nitrite and nitric oxide in the tissues and blood are to increase vasodilation, decrease blood pressure, support cardiovascular function and mitochondrial respiration, regulate renal hemodynamics, promote neurotransmission, exercise antimicrobial activity and exert a gastroprotective effect through enhanced motility and mucus production (Hord *et al.*, 2009, Albrecht *et al.*, 2003, Ralt, 2009, Sobko *et al.*, 2010).

Nitric oxide can be synthesized solely from arginine (Hord *et al.*, 2009) and oxygen. About 70% of nitric oxide is generated in the vascular epithelium by endothelial nitric oxide synthase (eNOS) with the two other NOS isoforms, nNOS (neuronal NOS) and iNOS (inducible NOS), generating variable amounts of nitric oxide in neuronal and chronically inflamed tissues, respectively. Because most enzymatically produced nitric oxide is retained in the tissues, enzymatic NO accounts for only about half of steady state nitric oxide with the remainder coming largely from the reduction of dietary nitrate to nitrite and then to nitric oxide (Hord *et al.*, 2009). Dietary nitrate is nearly 100% bioavailable to the plasma through the stomach and proximal small intestine (Hord *et al.*, 2009). About 25% of circulating nitrate is actively taken up by the salivary glands, concentrated and secreted in the saliva (0.2-2.5 mM), where about 20% of secreted nitrate (equivalent to 5-8% of ingested nitrate) is converted to nitrite (0.05-1 mM) by commensal anaerobic bacteria on the dorsal portion of the tongue (Pietraforte *et al.*, 2006, Sobko *et al.*, 2006, Takahama *et al.*, 2008). Swallowed along with unconverted salivary nitrate, newly formed nitrite is further reduced to nitric oxide through reaction with ascorbic acid supplied by the gastric juices of the stomach (Krul *et al.*, 2004, Moriya *et al.*, 2002) or commensal bacteria (*i.e.*, *Lactobacilli* and *Bifidobacteria*) in the intestines (Sobko *et al.*, 2006). Concentrations of salivary nitrate and nitrite are known to vary with recently ingested foods and with the use of antimicrobial mouthwashes (Hord *et al.*, 2009, Sobko *et al.*, 2010).

Dietary elevation of nitrate either through supplementation (potassium nitrate capsules delivering 4.13, 12.4 or 24.8 mg/kg bw/day) or through increased consumption of foods containing nitrate (250 ml *per* day beetroot juice delivering 5.8 mg/kg bw/day) has been shown to increase levels of circulating nitrite and nitric oxide in a dose-dependent manner in 21 healthy men and women (19-60 years of age) in three different studies, each lasting a maximum of 24 hours (Kapil *et al.*, 2010).

In a single-blind controlled cross-over study considering the effect of consuming commercially available fruit-and-vegetable-puree-based drinks on risk factors for cardiovascular disease (George *et al.*, 2009), total plasma nitrate and nitrite levels were found to increase significantly in the samples of 24 healthy participants (20 men, 4 women) after acute intervention (400 ml of pureed apple, carrot and strawberry containing 0.76 mg total nitrate and nitrite) when compared with samples from the sugar-matched control group for time points from 30-480 minutes after ingestion ($P < 0.05$ for all time points).

Elimination of nitrate and metabolites is primarily through the urine (65-70% of unchanged nitrate), although small amounts of nitrate are also excreted in (Sobko *et al.*, 2006, Saul *et al.*, 1981) and through breast milk (up to 5 mg/kg breast milk) (Sugekawa and Matsumoto, 1975). Because nitrate is physiologically necessary for appropriate functioning of the body, conservation mechanisms are in place in the small intestine and urinary tract for recycling nitrate; about 80% of the nitrate released into urine is actively transported back to the blood before voiding occurs (Kahn *et al.*, 1975).

8. SAFETY EVALUATION

8.1 Toxicity Studies

Creatine nitrate has not, to this point, been evaluated in a 90-day safety study in rodents or in any other animal. Individually, creatine and nitrate have been studied in animal models, but to varying degrees.

Although creatine has been the subject of several animal studies (*e.g.*, mouse, rat, guinea pig and dog at doses ranging from 0.05-2 g/kg bw/day for 14-96 days) (Ipsiroglu *et al.*, 2001, Ju *et al.*, 2005, Lowe *et al.*, 1998), few have focused on safety. No acute toxicity studies, for example, were located for creatine. The only creatine study in rats that indicated physiological toxicity used Sprague-Dawley (SD) rats (sex(es) not specified) having pre-existing cystic kidney disease (Edmunds *et al.*, 2001). Doses and exposure periods approximated human doses for loading (20 g/day or 0.3 g/kg bw/day for one week) and maintenance (5 g/day or 0.08 g/kg bw/day for five weeks). The resulting decrease in creatinine clearance with associated increases in kidney weight and serum urea together indicated compromised renal function in treatment group animals as compared to control group animals. In comparison, a supplementation study in rats with pre-existing renal failure induced by partial nephrectomy and given either a control diet or supplemented diet delivering 0.9 g/kg bw/day (approximating 50 g/day in a human) for four weeks showed no effect of creatine supplementation on creatinine or urea clearance or on urinary protein levels compared to sham operated rats given the control diet (Taes *et al.*, 2003). Due to the lack of defined adverse effects associated with creatine supplementation in animal studies, neither an LOAEL (lowest-observed-adverse-effect level) nor an NOAEL (no-observed-adverse-effect level) has been established. Without an LOAEL or NOAEL to support a Tolerable Upper Limit Uptake (UL) for creatine, the Institute of Medicine has not set an UL for creatine (Shao and Hathcock, 2006).

The acute oral toxicity of nitrate is low. In mice, rats, rabbits and pigs, oral LD_{50}[16] values of 2500-6250, 3300-9000, 1900-2680 and 300 mg/kg bw, respectively, have been reported (Walker, 1990, Speijers *et al.*, 1987). In humans, severe toxic effects are associated with oral doses above 10 grams[17] (166 mg/kg bw), with doses near 20 grams (~330 mg/kg bw) often being lethal (Walker, 1990).

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[16] LD_{50}= the dose that produces 50 *percent* lethality in the test population

One subchronic toxicity study performed in two dogs considered the effect of a diet containing 2% sodium nitrate (equivalent to 370 mg/kg bw/day nitrate) over periods of 105 and 125 days (Lehman, 1958). No adverse effects were observed in the dogs. When rats (number of animals not specified) were given drinking water containing up to 10% sodium nitrate over a six-week period, the only reported effect was a slight but not significant increase in methaemoglobin[18] (EFSA, 2008).

Chronic oral toxicity of nitrate is also low. In a two-year study by Lehman (1958, 1958), rats were fed 0, 50, 500, 2500 and 5000 mg/kg bw/day sodium nitrate (equivalent to 0, 36.47, 364.7, 1823.6 and 3647 mg/kg bw/day nitrate) *via* diet. Although no adverse histological changes or increases in tumor frequency were found in any diet group, the NOAEL[19] for sodium nitrate was 500 mg/kg bw/day due to depressed growth rates and the occurrence of inanition[20] among rats of the 2500 and 5000 mg/kg bw/day groups. In a second study where rats were dosed for 84 weeks with 0 and 500 mg/kg bw/day sodium nitrate (equivalent to 0 and 364.7 mg/kg bw/day nitrate) in drinking water (Lijinsky *et al.*, 1973), no histopathological effects were reported. In a third study (Maekawa *et al.*, 1982), rats were provided 0, 2500 and 5000 mg/kg bw/day sodium nitrate (equivalent to 0, 1823.6 and 3647 mg/kg bw/day nitrate) in drinking water for a two-year period. The only treatment-related effect identified was a slight to moderate reduction in body weight gain observed in 5000 mg/kg bw/day group rats; the oral NOAEL for sodium nitrate was 2500 mg/kg bw/day.

In summary, when considered as individual components, both creatine and nitrate are relatively nontoxic. When orally administered at levels approximating human usage (0.3 g/kg bw/day for seven days and 0.08 g/kg bw/day for 35 days), creatine has been associated with toxicity only in rats already having kidney disease. Due to the lack of defined adverse effects associated with creatine supplementation in conducted animal studies, neither a LOAEL nor a

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[17] <http://toxnet.nlm.nih.gov>; accessed for potassium nitrate (RN 7631-99-4) and sodium nitrate (RN 7757-79-1) October 5, 2010.

[18] One of two products formed from the reaction of oxyhaemoglobin with nitric oxide, nitrate being the second.

[19] NOAEL = No Observed Adverse Effect Level.

[20] Extreme exhaustion or lethargy, usually from a lack of nourishment.

NOAEL has been established. Nitrate, on the other hand, has oral LD_{50} values of 3300-9000 mg/kg bw in rats and is generally expected to cause lethality in humans at acute doses ≥ 330 mg/kg bw, similar to the oral LD_{50} value (300 mg/kg bw) observed in pigs. In two feeding trials using the rat model, NOAELs of 364.7 mg/kg bw/day and 1823.6 mg/kg bw/day were determined for nitrate in diet (for two years) and drinking water (for 84 weeks), respectively.

8.2. Genotoxicity studies

Intact creatine nitrate has not been evaluated for genotoxic or mutagenic potential. Individually, however, creatine and nitrate have been studied quite extensively over a period of years.

Cancer etiology became associated with creatine when it was discovered in the 1970s that cooking could generate heterocyclic amine (HA) carcinogens at ppb[21] (ng/g) levels in cooked meat (Murkovic *et al.*, 1998, Schut and Snyderwine, 1999) from the amino acids, creatine and sugars naturally present in the raw meats (Schut and Snyderwine, 1999, Felton and Knize, 2006). Formation of HAs has since been shown to be dependent on temperature (Reutersward *et al.*, 1987a, Reutersward *et al.*, 1987b, Yoshida and Okamoto, 1980), the presence of sufficient fat (Vithayathil *et al.*, 1983) and the presence of sugars, especially of glucose (Reutersward *et al.*, 1987a, Reutersward *et al.*, 1987b, Yoshida and Okamoto, 1980). The major HA species of concern were identified as the amino-imidazoazaarenes; 2-amino-3-methylimidazo[4,5-f]quinolone; 2-amino-3,4-dimethylimidazo [4,5-f]quinolone; 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx). Except for DiMeIQx, the other HAs have demonstrated carcinogenicity in animals (Schut and Snyderwine, 1999).

Observed carcinogenic activity was initially thought to be a function of creatine concentrations, but when the extracts from the fried meat of 15 different animals were tested using a modified Ames assay with *Salmonella typhimurium* TA98 in the presence of S9 activation to evaluate mutagenicity, mutagenic activity did not directly correlate with creatine/creatinine concentrations (Vikse and Joner, 1993). These findings were in agreement with a similar evaluation in seven species of fish commonly consumed in the Hawaiian Islands

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[21] ppb=parts *per* billion

(Marsh *et al.*, 1990), where again mutagenic activity was not directly related to creatine concentration. Additionally, a five day supplementation study (up to 50 g CreM *per* day) in two breeds of domestic pigs (*i.e.*, Duroc and Landrace) showed no effect of supplementation on HA generation in the fried meats or on mutagenicity in the meat extracts (Pfau *et al.*, 2006). Although creatine and creatinine are *N*-nitroso-precursors generally consumed by humans at levels approaching 1 g/day, these substances are not the most abundant nitrosatable components in the diet; amides in the form of proteins are consumed at levels approaching 100 g/day and have been proposed as the substances primarily responsible for the formation of HAs (Shephard *et al.*, 1987).

Although the formation of *N*-nitrososarcosine, a known carcinogen, from ingested creatine after exposure to the high acidity and high nitrite levels found in the stomach has periodically been voiced as a concern (Archer, 1999, Archer, 2004a, Archer, 2004b), a double-blind placebo controlled study by Derave *et al.* (2006) conducted in healthy male and female volunteers demonstrated that excessive creatine loading (>20 g/day) did not increase urinary *N*-nitrososarcosine concentrations. Rather than promote mutagenesis, creatine supplementation has recently been demonstrated to normalize mitochondrial DNA mutation and to have a potential role in preserving mitochondrial functionality (Guidi *et al.*, 2008, Berneburg *et al.*, 2005). Further, when administered along with methylglyoxal in cancer-bearing mice, creatine has been found to significantly augment the anticancer effect of methylglyoxal resulting in increased lifespans and inhibited tumor growth for Ehrlich ascites carcinoma cells (Ghosh *et al.*, 2006)

As determined by use of *in vitro* testing, sodium nitrate is not mutagenic (NTP, 2001). For nitrite, however, mutagenic potential was found for *Salmonella typhimurium* strain TA100 with and without metabolic activation but not for *S. typhimurium* strain TA98 (NTP, 2001). When the ability of nitrate and nitrite to produce chromosome aberrations in mammalian cells were evaluated using *in vitro* cultures of peripheral blood lymphocytes (Balimandawa *et al.*, 1993), aberrations did not increase with sodium nitrate doses (17.6-70.6 mM) but did increase slightly (*P* values not specified) in both micronucleated cells and chromatid gaps for sodium nitrite (14.4 mM). When further examined using *in vivo* rodent models (*i.e.* micronuclei induction in the bone marrow of rats and mice or in peripheral blood for mice), no effects were found for sodium nitrite which suggested that the substance was not genotoxic *in vivo* (NTP, 2001, FAO/WHO, 2003a, FAO/WHO, 2003b). Although no significant increase in the mean number of sister chromatid exchanges *per* cell occurred in a group of Greek children (*n* = 70; 12-15 years of age) exposed to nitrate concentrations above 70.5 mg nitrate/L through local drinking waters compared to a control group (*n* = 20) exposed to low nitrate concentrations (*i.e.*, 0.7 mg/L), a significant increase in the mean number of chromatid/ chromosome breaks was reported ($P < 0.01$) (Tsezou *et al.*, 1996). Overall, the JECFA concluded that there was no evidence for the reclassification of either nitrate or nitrite as genotoxic compounds (FAO/WHO, 2003a, FAO/WHO, 2003b). In agreement with the conclusions drawn by JECFA, a review by Milkowski *et al.* (2010) considering the risks and benefits of dietary nitrate and nitrite in the context of nitric oxide biology in human health determined that the data supporting an increased cancer risk from dietary nitrate and nitrite was weak and inconclusive: "Firm evidence of cancer causation in humans is lacking for dietary nitrite and nitrate." The authors also noted that an earlier comprehensive review by Eichholzer *et al.* (1998) did not find any "epidemiological evidence linking stomach, brain, esophageal and nasopharyngeal cancers to dietary intake of nitrate, nitrite or *N*-nitroso compounds."

In summary, although both creatine and nitrate have been/are associated with substances that form mutagenic entities, neither is genotoxic in and of itself. Further, the two-year study by Lehman (1958, 1958) demonstrated that rats fed up to 5000 mg/kg bw/day sodium nitrate (equivalent to 3647 mg/kg bw/day nitrate) exhibited no adverse histological changes or increases in tumor frequency compared to the control rats. Both creatine and nitrate are substances critical to the health of the organism and the body has substantial resources in place to ensure against the depletion and/or unnecessary excretion of either.

8.3. Additional preclinical studies

No studies have been published about the carcinogenicity, developmental or reproductive toxicity of CreN. Creatine and nitrate have, however, been studied as independent substances.

In a 40-day study in rats by Horn *et al.* (1998), the effect of increased dietary creatine intake on total creatine concentrations in the heart, skeletal muscle, brain, liver, kidney and serum were investigated. Wistar rats (~450 g; sex(es) not specified) were assigned to one of five feeding groups: (1) creatine-free negative control diet (*n* = 15), (2) creatine-free feed with 1% CreM by weight (*n* = 12), (3) creatine-free feed with 3% CreM (*n* = 18), (4) creatine-free feed with 5% CreM (*n* = 15), and (5) creatine-free feed with 7% CreM (*n* = 17). Diets containing up to 3%

CreM had no effect on feed consumption or body weight over a 40-day period. The body weights of animals on diets containing 5% and 7% CreM, however, were reduced by 10% and 13%, respectively, compared to control ($P < 0.05$ for both *via* ANOVA[22]), but without discernible effect on feeding behavior. Serum total creatine concentrations in all diet groups increased significantly compared to control (Scheffe's *F*-test). Total creatine content was not, however, altered in the heart myocardium and brain in any dietary group; neither was there a change in the total creatine content of skeletal muscle up to the 3% diet, the highest diet group evaluated for skeletal muscle creatine content. In liver and kidney tissues of the 3%, 5% and 7% diet groups, total creatine was significantly increased compared to the control group (Scheffe's *F*-test). The authors concluded that the chronic administration of large amounts of dietary creatine could increase concentrations of total creatine in the blood by about three-fold and promote accumulation in the liver and kidney, but not in the heart, brain or skeletal muscle. The results of this study were noted by the authors as being in disagreement with the results of a study by (Harris *et al.*, 1992) which showed that dietary supplementation in humans can increase total creatine content of skeletal muscle by 17%. No adverse effect of supplementation in the rats was reported. A later study (Ju *et al.*, 2005) in 14 female Wistar rats (~90 g) receiving control feed ($n = 7$) or feed supplemented with CreM (2% of diet; $n = 7$) for three weeks, found no significant change in creatine concentration of the extensor digitorum longus (EDL) muscles (6.8 ± 0.7 μmol/g *v.* 6.1 ± 0.4 μmol/g control), but a significant increase in creatinine content in EDL muscle from supplemented rats compared to content in EDL from the control group (2.9 ± 0.3 μmol/g *v.* 1.6 ± 0.2 μmol/g control; $P < 0.005$). Again, no creatine-related adverse effect was identified in treatment group animals.

To investigate the effects of CreM supplementation on growth performance (Moghadam *et al.*, 2008), 36 young male Sprague-Dawley rats and 36 Ross 308 chickens (35-day old) were separated into three equal groups ($n = 12$/group). The control group received water without supplementation. The experimental groups received CreM at doses of 250 and 500 mg/kg bw/day for 15 days in water. Hyperactivity and increased aggressiveness occurred in CreM-supplemented rats after Day 7; no change in behavior was observed in the chickens. At the end of 15 days, no effect on body weight or feed conversion was found between control and dose group rats. For chickens, the feed conversion rate did not change but body weight increased significantly at day 15 for the 500 mg/kg bw/day group compared to the control group ($P < 0.05$). No significant histopathological changes in liver, kidney or skeletal muscle were identified. The authors concluded that creatine improved body weight gain in chickens without exerting a pathological effect on liver, kidney or muscle and that no adverse effect was identified in chickens or rats with supplementation, other than an increase in activity and aggressiveness in the rats. An increase in aggressiveness is anticipated with increasing creatine kinase concentrations and has been observed even in human males (Hillbrand *et al.*, 1998).

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[22] Analysis of variance

The effect of chronic creatine loading and voluntary wheel running on the speed and contractile properties of the myosin heavy chain (MHC) isoform profile in the rat mixed fast-twitch plantaris was explored in a 13-week study which also examined the fast-twitch extensor digitorum longus and medial gastrocnemius and the slow-twitch soleus (Gallo *et al.*, 2006). Forty SD male rats (three weeks old) were assigned to one of four groups after classification as high (willing) or low (less willing) runners. Low runners were assigned to creatine-sedentary (Cre-Sed) and control-sedentary (Con-Sed) groups; willing runners were assigned to creatine- and control-voluntary running (Cre-Run and Con-Run, respectively) groups. The drinking solution for the control groups consisted of a 5% dextrose solution; the test solution contained 1% creatine in a 5% dextrose solution. Food intake was an average of 20-30% higher in the running groups compared to the sedentary rat groups. Fluid intake also averaged 25% higher in the running compared to the sedentary groups which indicated the average creatine ingested on a daily basis was also about 25% higher in the running groups (3000 ± 140 mg/kg bw/day) compared to the sedentary groups (2400 ± 170 mg/kg bw/day; $P < 0.02$). Creatine dosing was not found to affect daily run distances or total run distances between the run groups. Body weights for rats in all groups were similar until Week 9, when running began to demonstrate an effect; at the end of the study, Cre- Run and Con-Run group rats averaged 14% and 17%, respectively less weight than the corresponding sedentary groups ($P < 0.0002$). Interestingly, the mass-to-body mass ratio for each of the muscles examined was similar among all groups. When examined in the plantaris muscle, neither running nor creatine was found to affect muscle hydration.

Although contractile properties[23] did not differ for contraction or relaxation speed in any muscle among the study groups, isometric force production[24] was found to vary between the soleus and plantaris, but only for tetanic force between the Cre-Sed and Con-Sed groups ($P < 0.05$ for both). The fatigue index at ten seconds (FI10) indicated the Cre-Run group plantaris was less fatigued than the Con-Run group plantaris ($P < 0.05$). For the other muscle groups, Cre-Sed was less fatigued based on FI10 than Con-Sed ($P < 0.05$ for both). For the fatigue index at 30 seconds (FI30), Cre-Sed was less fatigued than Con-Sed in soleus and extensor digitorum longus ($P < 0.05$ for both). Prolonged creatine supplementation did not affect the ATP/ADP ratio or the content of high energy phosphoryls in the medial gastrocnemius; in the plantaris, only ADP free was significantly greater in the Cre-Run group than in its respective control group ($P < 0.05$). Total creatine was significantly higher in plantaris muscle of the Cre-Run group compared with the Con-Run group ($P<0.03$). Myosin heavy chain (MHC) isoform content was affected by supplementation or running only in the plantaris muscle. In Cre-Run group rats compared to the Con-Run group, MHCIIa and MHCIId/x contents significantly decreased ($P < 0.0002$ for both) while MHCIIb increased ($P < 0.00001$). In Cre-Sed group rats compared to the Con-Sed group, MHCIId/x content significantly increased ($P < 0.0001$) while MHCIIb was decreased ($P < 0.0006$). MHCI content was not affected by supplementation or running in the plantaris. The authors concluded that "Creatine supplementation had differential effects on MHC isoform content and fatigability that depended on the level of contractile activity. Creatine feeding combined with running exercise resulted in a faster MHC-based phenotype in the rat plantaris but the impact on associated isometric contractile properties was minimal." No adverse effects with supplementation were reported.

In a subsequent 13-week study to understand how creatine loading resulted in a higher proportion of MHC IIb in the plantaris muscle without affecting the contractile properties (Gallo *et al.*, 2008), 40 SD male rats (three weeks old) were assigned to one of four groups (Cre-Sed, Cre-Run, Con-Run, Con-Sed) and were dosed and exercised as previously described (Gallo *et al.*, 2006). No changes were observed in the percentage of plantaris muscle fibers expressing MHC I or the various hybrid fiber types. The percentage of fibers expressing MHCIIa increased significantly with running in the Cre-Run and Con-Run groups compared to the Cre-Sed ($P < 0.04$) and Con-Sed ($P < 0.0003$) groups, respectively, but with a substantially smaller increase for the creatine groups compared to the Con groups ($P < 0.02$). In contrast, the expression of MHCIIb significantly decreased with running in Con-Run compared to Con-Sed ($P < 0.004$) with Cre-Sed and Cre-Run expression comparable to Con-Sed. For MHCIId/x expression, the proportion of fibers decreased in the Cre-Run ($P < 0.03$) and Con-Run ($P < 0.05$) compared to their respective sedentary controls. In general, only running was associated with increases in the cross-sectional areas (CSA) of pure fiber and hybrid IIa/IId(x) fibers. The CSA of type I, I/IIa, IId(x), and IIb fibers in Cre-Sed increased significantly compared to Con-Sed independent of running ($P < 0.002$). In the running groups, however, CSA of type IIa/IId(x), IId(x), and IIb fibers significantly decreased in Cre-Run compared to Cre-Sed ($P < 0.002$). Parvalbumin content decreased significantly in the creatine groups compared to the control groups ($P < 0.01$) and in the run groups compared to their respective sedentary groups ($P < 0.05$ for both). In comparison, the fast Ca^{2+}-ATPase (SERCA1) isoform present in the plantaris muscle did not vary with creatine or running. The slow isoform (SERCA1), however, increased in response to running alone ($P < 0.005$) and to creatine alone ($P < 0.03$) and further increased for both running and creatine ($P < 0.02$). Although associated enzyme activities for phosphofructokinase and glyceraldehyde-phosphate dehydrogenase were not affected, 3-hydroxyacyl-CoA dehydrogenase and citrate synthase activities did increase significantly in the Con-run group compared to the Con-Sed group ($P < 0.05$ and $P < 0.0001$, respectively). The adaptive changes indicate that the need for intracellular Ca^{2+} buffering by parvalbumin is reduced and the efficiency of Ca^{2+} uptake by SERCAs is increased when the capacity for high energy phosphoryl shuttling *via* creatine is increased. The authors concluded that creatine loading during run training supports a faster muscle phenotype that is adequately supported by the existing glycolytic potential, without requiring adaptive changes in the capacity for terminal substrate oxidation.

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[23] Time-to-peak tension, half-rise time, half-time for muscle relaxation

[24] Twitch force, tetanic force, sag ratio

Other rat studies have demonstrated that creatine supplementation 0.2-5 g/kg bw/day for periods of five days to six months) attenuates the muscle-wasting effects of immobilization (Aoki *et al.*, 2004), enhances creatine disposal and glycogen storage in skeletal muscle (Op 'T Eijnde *et al.*, 2001), upgrades the functionality of surgically reinnervated subjects (Özkan *et al.*, 2005), significantly reduces plasma homocysteine levels in comparison to groups on guanidinoacetate-supplemented or control diets ($P < 0.05$; (Stead *et al.*, 2001)), increases survival time and decreases the cytotoxic effects associated with doxorubicin treatment (Santos *et al.*, 2007) , improves fatigue resistance in soleus muscles but not in respiratory muscles of the diaphragm (McGuire *et al.*, 2002), and increases the energetics of the myocardium similar to effects observed in skeletal muscle (Brzezińska *et al.*, 1998), but is unable to diminish post-myocardial infarction functional and energetic derangement of the heart (Horn *et al.*, 1999, Horn *et al.*, 1999). In none of the studies were adverse effects related to creatine supplementation reported.

Regarding nitrate, one rodent study has demonstrated that nitrate supplementation (0.3 g/kg bw/day for a period of seven days) exerts a protective effect on gastric mucosa through the production of nitric oxide in an iodoacetamide-induced gastritis rat model (Larauche *et al.*, 2003). Another has demonstrated that dietary deficiency of nitrate and nitrite in mice effectively diminishes circulating and tissue stores of nitrite and nitric oxide which results in a 59% increase in the severity of myocardial ischemia-reperfusion injury (Bryan *et al.*, 2007). The effects of deficiency were reversed by adding nitrite to the drinking water for seven days. No adverse effects related to the ingestion of nitrate or nitrite supplementation were reported in any subjects.

8.4. Observations in Humans

Although there is no record in the scientific literature of CreN use by humans, creatine and nitrate have been separately used by and studied in humans.

8.4.1. Observations in Humans-Creatine

No acute overdose data is available for creatine, neither is there an established toxic dose although it has been suggested that doses > 2 g/individual/day (> 33 mg/kg bw/day) could induce renal dysfunction (HSDB, 2010). In humans, thus far, no controlled studies have revealed severe adverse effects associated/caused by creatine supplementation. Renal function, tested in human subjects taking creatine in doses ranging from 1-80 g/day (16-1333 mg/kg bw/day) for periods of five days up to five years was not found to be adversely affected (Poortmans *et al.*, 1997, Poortmans and Francaux, 1999, Poortmans and Francaux, 2000). There are, however, two case studies in which kidney function and health were negatively affected by creatine supplementation at high load levels. A previously healthy man developed interstitial nephritis (Koshy *et al.*, 1999) and kidney function began to deteriorate in a young man having focal segmental glomerulosclerosis (Pritchard and Kalra, 1998). In both cases renal function recovered with cessation of supplementation. Creatine supplementation is currently contraindicated in individuals with renal failure and/or renal disorders such as nephritic syndrome (PDR, 2001)

In contrast to the above case reports, a 20 year old man having a single kidney with mildly decreased glomerular filtration rate underwent a 35-day creatine supplementation trial to improve strength and muscle mass (Gualano *et al.*, 2010). The supplementation regimen consisted of 20 g/day (0.29 g/kg bw/day) in four equal doses for five days followed by 5 g/day in a single dose (0.07 g/kg bw/day) for 30 additional days. During this same period he also consumed a high protein diet with an average intake of 2.8 g/kg bw/day (approximately three-fold the recommendation for healthy individuals). In samples collected just after the intervention compared to baseline samples, serum creatinine concentrations increased slightly but not significantly (1.03 mg/dL baseline *vs.* 1.27 mg/dL); the creatine concentrations were still within the normal range of 0.57-1.30 mg/dL (Shao and Hathcock, 2006), while 24-hour albuminuria, serum urea and estimated creatinine clearance[25] were decreased (4.6 mg/d baseline vs. 2.9 mg/d; 37 mg/d baseline vs. 28 mg/dL; and 88 mL/min/1.73 m^2 baseline vs. 71 mL/min/1.73 m^2, respectively). No effect was observed for ^{51}Cr-EDTA clearance (pre, 81.6 mL/min/1.73 m^2; post, 82.0 mL/min/1.73 m^2), 24-hour proteinuria (protein excretion (130 mg/d baseline vs. 120 mg/d) or for the serum and/or urinary electrolytes (*i.e.*, sodium and potassium). The authors concluded that "short-term creatine supplementation may not affect kidney function in an individual with a single kidney, mild decreased GFR, and ingesting a high-protein diet (*i.e.*, 2.8 g/kg/d)."

In their risk assessment for CreM, Shao and Hathcock (2006) recommended an Observed Safety Limit (OSL) of 5 g/day for humans, based on an assessment of 22 double-blind, randomized, controlled human clinical trials including both acute (high doses of 20 g/day or so over short intervals of not more than one week) and chronic (3-5 g/day for periods of up to one year) studies, found in Appendix 1. Based on their assessment, the authors determined that the available studies in the scientific literature demonstrate safety for ingested creatine at levels up to five grams *per* day under chronic administration. The authors also determined that although creatine is frequently administered at higher dose levels for short periods of time, safety was not sufficiently proven under controlled conditions at these levels for additional conclusions to be made at this time.

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[25] Calculation based on Cockcroft-Gault equation.

Among athletes and bodybuilders, creatine is a readily available and popular ergogenic aid and performance enhancer. Supplementation has been shown to increase available stores of creatine and PCre in skeletal muscle for use during short-term intermittently strenuous activities (*e.g.*, repetitive running sprints and high intensity bouts of cycling), but not during long-term aerobic activities (*e.g.*, endurance running or cycling) (Fernandez and Hosey, 2009, Derave *et al.*, 2006, Juhn, 2003). Widely believed to increase muscle mass, increases averaging 1.0-2.3% in fat-free skeletal-muscle mass are supported by the scientific literature (Francaux and Poortmans, 2006). The typical recommended supplemental regimen originally consisted of maximal daily dosages of up to 20 grams for "loading" over a short term period such as five days (Wyss, 2004) with two to five grams taken later for maintenance over extended periods of time (Juhn, 2003, Anonymous, 2004). Rather than loading over a period of days, current supplementation strategies often recommend a single dose of up to five grams of creatine one hour prior to or after exercise, an approach which is now recognized to increase uptake by about ten *percent* (Shao and Hathcock, 2006). Two grams of creatine *per* day have been demonstrated as sufficient to maintain maximum creatine concentrations in skeletal muscle (Hultman *et al.*, 1996). Despite recommendations, professional and amateur athletes frequently use larger doses (Taes and De Vriese, 2005).

Even though creatine supplementation is known to have a highly individualized effect in performance, two effects have been consistently documented, modest weight gain (1-5 kg; (Galbraith *et al.*, 2006)) and an increase in muscle compartment pressure. Both are thought to be associated with intracellular water retention (Juhn, 2003, Anonymous, 2004, Mesa *et al.*, 2002, Schroeder *et al.*, 2001). After 5-14 days of supplementation at a rate of 20-25 g/day, skeletal muscle mass has been shown to increase with coinciding enhancement in the performance of intermittent high intensity exercise (Williams and Branch, 1998). The effects of creatine supplementation tend to be most apparent in vegetarians (having low previous creatine intakes) and sedentary untrained subjects rather than in trained athletes who do not seem to benefit to the same degree (Williams and Branch, 1998). Cellular hydration is usually indicative of protein synthesis when anabolics are present; the increased cellular hydration associated with creatine consumption has not yet been demonstrated to upregulate the active building of muscle tissue, however (Moghadam *et al.*, 2008, Juhn, 2003).

In addition to the previously mentioned concerns regarding safety (*i.e.,* nephrotoxicity), a number of mild to moderate adverse side effects from general-use creatine supplementation have also been anecdotally reported (*i.e.*, heat intolerance, muscle cramps and muscle strains). In comparison, anecdotal side effects specific to creatine use in the pursuit of athletic enhancement include nausea, vomiting, diarrhea, elevated serum transaminase concentrations, hypertension, fluid retention, muscle cramping and muscle strain (Chyka, 2003). The consumption of creatine quantities not normally found in food sources has been mentioned as a concern regarding side effects. Considering that the levels of creatine consumed by some athletes may exceed by greater than 10-fold the 2 to 4 grams endogenously generated and consumed through a normal daily diet, the potential for side effects is not surprising. The findings of clinical trials studying athletes and military personnel with short and long term supplementation (predominately of CreM), however, have supported safe usage (Shao and Hathcock, 2006, Poortmans and Francaux, 1999, IOM, 2008). Overall, the main toxic side effect consistently reported during the studies has been short term gastrointestinal distress (*i.e.*, abdominal cramping, nausea, vomiting and diarrhea) due to malabsorption of high doses, generally > 20 g/day (Francaux and Poortmans, 2006, Shao and Hathcock, 2006, HSDB, 2010, IOM, 2008).

To evaluate the effects of long-term creatine supplementation on the clinical health status of athletes (18-23 years of age) (Kreider *et al.*, 2003), treatment group participants received doses of 15.75 g/day (262.5 mg/kg bw/day) of CreM for five days, then an average of 5 g/day (83.3 mg/kg bw/day) in 5-10 g/day (83.3-166.7 mg/kg bw/day) doses over a maximum period of 21 months in a non-blinded study. The study groups included negative control (no creatine; *n* = 44), 0-6 month supplementation (*n* = 12), 7-12 month supplementation (*n* = 25) and 12-21 month supplementation (*n* = 17). Clinical chemistry assessments included baseline and study period urine samples to monitor/establish clinical status and renal function and serum and whole blood samples to evaluate changes in metabolic markers, muscle and liver enzymes, electrolytes, lipid profiles, haematological markers, and lymphocytes. No significant difference was identified among the groups for any of the 54 monitored parameters. The authors concluded that long-term creatine supplementation caused no adverse effect among athletes in active training.

In athletes during maximal exercise, fatigue onsets partially from a depletion of PCre and the inability of PCre hydrolysis to maintain a high ATP: ADP ratio (Moghadam *et al.*, 2008). During high intensity exercise, muscle stores of PCre deplete within ten seconds. Initially, PCre buffers ATP hydrolysis *via* reaction with creatine kinase. At this stage, PCre is instantaneously available for ATP regeneration. In comparison to PCre which is instantaneously available for ATP regeneration, glycolysis and mitochondrial oxidative phosphorylation are both delayed by multiple seconds. To replenish muscle stores and delay depletion, creatine is often ingested by non-endurance athletes for a period of short-term loading (Moghadam *et al.*, 2008). In one study focusing on endurance athletes, significantly lower elevations in the pro-inflammatory cytokines (TNFα, INFα, IL-1β) and prostaglandin E_2 generated during a half-iron man competition has also been observed in human athletes who ingested 20 g/day creatine for five days prior to the event ($n = 5$) compared to those who loaded on 20 g/day carbohydrates ($n = 6$; $P < 0.05$ for all) (Bassit *et al.*, 2008). Only concentrations of IL-6 were unaffected. The effects were most pronounced 48 hours after the competition when delayed onset muscle soreness has typically onset. No adverse effects were reported in any participants.

In summary, although a few isolated case studies have implicated creatine supplementation as being nephrotoxic (Koshy *et al.*, 1999, Pritchard and Kalra, 1998, Kuehl *et al.*, 1998), the implication has not been supported in large studies including healthy humans (Kreider *et al.*, 2003, Farquhar and Zambraski, 2002). The most consistently reported side effects during formal studies are temporary gastrointestinal complaints and these are most generally experienced at dose levels in excess of 20 g/day (333 mg/kg bw/day). The necessity of adequate creatine stores for basic health is also demonstrated by the rarity and severity of creatine deficiency syndromes in young humans. Long term usage of creatine in military personnel and in athletes during controlled studies has not indicated toxicity with normal usage, loading doses of up to 20 g/day over short intervals of not more than one week and chronic or maintenance doses of 3-5 g/day for periods of up to one year.

8.4.2. Observations in Humans-Nitrate

In a randomized cross-over-designed intervention study, the effects of dietary nitrate were evaluated in 25 Japanese volunteers (36 ± 10 mean years of age; 10 men, 15 women) (Sobko *et al.*, 2010) consuming either a Japanese diet (which included an assortment of traditional vegetables) or a Western diet (which included items such as yogurt, corn flakes and sandwiches). Each diet was consumed for ten days with no wash-out period between diet phases. During the Japanese diet phase, consumption of nitrate was approximately 18.8 mg/kg bw/day, considerably greater than the ADI for nitrate in Japan (3.7 mg/kg bw/day). The Western control diet was designed to provide approximately 3.7 mg/kg bw/day nitrate. No significant changes in body weight were observed during the total study period of 20 days. At the end of the Japanese diet phase, plasma nitrate and nitrite levels were significantly higher than levels measured at the end of the Western diet phase ($P < 0.001$ and $P < 0.01$, respectively), as were the fasting salivary nitrate and nitrite levels after the Japanese diet phase compared to the Western control phase levels ($P < 0.001$ and $P < 0.01$, respectively). Although no significant change in systolic blood pressures was measured after the diet phases, diastolic blood pressures were significantly decreased after the Japanese phase compared to the Western phase ($P < 0.01$). No adverse effects associated with high dietary nitrate were reported.

In a randomized double-blind placebo-controlled crossover study (Larsen *et al.*, 2007), the effects of dietary nitrate on oxygen cost during exercise were examined in nine athletic young men (28 ± 6 years). During two three-day study periods the subjects avoided foods with moderate or high nitrate content in addition to alcohol and tobacco and were given either 0.1 mmol sodium nitrate/kg bw/day (equivalent to 6.2 mg/kg bw/day nitrate) in water as the treatment regimen or 0.1 mmol sodium chloride/kg bw/day in water as the placebo. The nitrate ingested in the treatment arm approximated the amount of nitrate consumed in 150-200 g for spinach, beetroot or other nitrate-rich vegetable. Between study diets, there was a ten-day wash out period. For the testing phase, the subjects pedaled an electrically braked cycle ergometer through consecutive submaximal (five 5-minute cycling stages at rate levels of 45, 60, 70, 80 and 85% VO_{2peak}[26] without rest between stages) and maximal (cycling to exhaustion against increasing resistance) exercise sessions. Resting systolic and diastolic blood pressures were both significantly lower after nitrate supplementation compared to after the placebo ($P < 0.01$ for both). Neither hematocrit nor hemoglobin concentrations were affected by treatment or by exercise (pre-test *vs.* post-test). Plasma nitrite levels were significantly increased with nitrate supplementation compared to control ($P < 0.01$) prior to maximal exercise. When measured after maximal exercise the nitrite levels for the two diet periods were not significantly different - the decreases in nitrite levels after the supplemented period were more extensive than those after the placebo period. Although supplementation did not affect heart rate, blood lactate levels, pulmonary ventilation, CO_2 output or respiratory exchange ratio during submaximal exercise, VO_2 significantly decreased with nitrate supplementation compared to the placebo ($P < 0.01$) and gross efficiency significantly increased ($P < 0.01$). None of the measured parameters demonstrated a significant effect with maximal exercise. No adverse effects were reported among the volunteers.

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[26] Peak oxygen uptake

In a later study examining the effect of increased dietary nitrate on maximal oxygen consumption during maximal exercise, Larsen *et al.* (2010) conducted a randomized double-blind placebo-controlled crossover study in nine athletic male and female volunteers (30 ± 2.3 years). During two 2-day study periods the subjects avoided foods with moderate or high nitrate content in addition to alcohol and tobacco and were given either 0.1 mmol sodium nitrate/kg bw/day (equivalent to 6.2 mg/kg bw/day nitrate) in water as the treatment regimen or 0.1 mmol sodium chloride/kg bw/day in water as the placebo. Between the study diets, there was a seven-day wash out period. For maximal exercise testing, the subjects pedaled or pedaled and cranked an electrically braked arm and leg ergometer to exhaustion or until cadence dropped below 70 rpm (rotations *per* minute). Prior to exercise neither resting systolic nor diastolic blood pressures showed an affect with diet. Two minutes after exercise diastolic blood pressure was significantly decreased for the nitrate diet compared to the placebo diet ($P < 0.05$). The plasma concentrations of cyclic guanosine monophosphate, renin, aldosterone, and 20 different amino acids were not affected by nitrate supplementation at rest or after exercise. Post-exercise, no effect of supplementation was found on heart rate, pulmonary ventilation or lactate formation. Compared to exercise after the placebo diet, VO_2 decreased significantly post-supplementation ($P < 0.05$) and the time to exhaustion gave a nearly significant increase. The authors concluded that the decrease in VO_2 with nitrate supplementation indicated a real increase in performance. No adverse effects with supplementation were reported among the male and female volunteers.

In a similarly designed randomized double-blind placebo-controlled crossover study (Bailey *et al.*, 2009), the effect of inorganic nitrate from beetroot juice on oxygen cost during submaximal exercise and tolerance to high-intensity exercise was investigated in eight athletic male volunteers (26 ± 7 years). For the treatment arm, subjects consumed 500 ml of beetroot juice (delivering 5.79 mg/kg bw/day nitrate) each day for six days. For the placebo arm 500 ml of blackcurrant cordial (having negligible nitrate content) were consumed on each of six days. Moderate and severe intensity exercise tests (*i.e.*, pedaling an electrically braked cycle ergometer) were conducted on each of the last three days of the diet phase. Compared to the placebo phase, plasma nitrite was significantly greater ($P < 0.05$) and systolic blood pressure was significantly reduced ($P < 0.01$) after nitrate supplementation. With submaximal exercise, VO_2 was significantly reduced post-nitrate supplementation compared to placebo ($P < 0.05$). With maximal exercise, VO_2 was significantly reduced and the time to exhaustion was increased after nitrate supplementation compared to placebo ($P < 0.05$ for both). The findings of this study regarding the effect of nitrate supplementation on oxygen utilization were in agreement with those studies by Larsen *et al.*, (2007, Larsen *et al.*, 2010) that provided nitrate supplementation *via* a synthetically prepared material. Again, no adverse effects were reported in any volunteer.

In summary, various dietary supplementation approaches to increase nitrate (*i.e.*, high nitrate vegetables, potassium or sodium nitrate, vegetable and fruit purees, and beetroot juice) in healthy men and women and male and female athletes were shown to be increase blood nitrite levels and also shown to decrease blood pressure and improve oxygen utilization and exercise efficiency without increasing blood lactate levels. Adverse effects were not reported in any of the studies.

9. EVALUATION

Creatine nitrate is a white crystalline powder manufactured from food-grade creatine monohydrate reacted with nitric acid and ethanol. The ionic bonding of creatine (a nitrogenous organic acid produced endogenously in the liver, pancreas, and kidneys and consumed from high-protein dietary sources such as meat and fish) and nitrate (a common constituent in vegetables and recognized precursor to nitric oxide) prevents the association of water found in the common creatine monohydrate formulations. As a nitrate salt, CreN disassociates rapidly upon ingestion, releasing creatine which is necessary for the production of energy within muscular tissues, especially under short term high stress situations such as high-intensity exercise, and nitrate, precursor to nitric oxide, the latter of which is the major vasodilator in the body. On a daily basis, 1-2 grams (16-33 mg/kg bw/day) of creatine are generated endogenously with an additional 1-2 grams coming from dietary sources. In comparison, only small amounts of nitrate are produced endogenously (from arginine) while a majority of nitrate is obtained from the diet; in the US this estimate is 40-100 mg/day (0.66-1.66 mg/kg bw/day).

Although no studies have been conducted in animal models for CreN, when considered as individual components, both creatine and nitrate have been shown to be relatively nontoxic. When orally administered at levels approximating human usage (300 mg/kg bw/day for seven days and 80 mg/kg bw/day for 35 days), creatine has been associated with toxicity only in rats already having kidney disease. In other studies conducted in mouse, rat, guinea pig and dog models where creatine doses ranged from 50-2000 mg/kg bw/day for 14-96 days, no toxicity was identified. Nitrate which has oral LD_{50} values of 3300-9000 mg/kg bw in rats is generally expected to cause death in humans at acute doses of in excess of 20 grams (~330 mg/kg bw), similar to the oral LD_{50} value (300 mg/kg bw) observed in pigs. In two feeding trials using the rat model, NOAELs of 364.7 mg/kg bw/day and 1823.6 mg/kg bw/day were determined for nitrate in diet (for two years) and drinking water (for 84 weeks), respectively.

In humans, especially among the military personnel and athletes, creatine supplementation has been used as an ergogenic aid and performance enhancer starting in the 1970s. The traditional supplementation regimen begins with maximal daily dosages of up to 20 grams (equivalent to 333 mg/kg bw/day) for "loading" over a short period, usually five to seven days, with 2 to 5 grams (33-83 mg/kg bw/day) taken over extended periods of time for "maintenance" after the loading period. Because current supplementation strategies recognize that two grams of creatine *per* day are sufficient to maintain maximum creatine concentrations in skeletal muscle and that exercise increases the efficiency of uptake into the muscle tissue, a single dose of up to 5 grams of creatine (equivalent to 83 mg/kg bw/day) one hour prior to or after exercise is often recommended. Despite recommendations, professional and amateur athletes frequently use larger doses. Although a number of mildly to moderately adverse side effects from creatine supplementation in humans have been anecdotally reported (*i.e.*, heat intolerance, muscle cramps and muscle strains) in addition to concerns regarding safety (*i.e.*, nephrotoxicity based on two case studies), the findings of clinical trials studying athletes and military personnel with short and long term supplementation (predominately of CreM) have supported safe usage. Overall, the main toxic side effect consistently reported during human studies has been short term gastrointestinal distress (*i.e.*, abdominal cramping, nausea, vomiting and diarrhea), generally with doses of creatine in excess of 20 g/day (>333 mg/kg bw/day). The studies examining the effects of nitrate in humans have used dosage levels in the 4.13-24.8 mg/kg bw/day range for 24-hour to 10-day periods. In none of these studies were adverse effects reported.

Creatine nitrate as a dietary supplement would provide an additional source of creatine and nitric oxide (from nitrate) in the diet. Taken with 8 ounces of water two times *per* day, 30 minutes prior to exercise and within one hour following exercise, the supplement would provide a total of 2037 mg creatine *per* day (33.95 mg/kg bw/day) and 963 mg nitrate *per* day (16.05 mg/kg bw/day) to the diet in two servings of 1500 mg CreN. Based on the available data, including *in vivo* testing in animal models, genotoxicity studies, and information from clinical trials for creatine and nitrate as independent substances, creatine nitrate is reasonably expected to be safe when used as a dietary supplement under the recommended conditions of use.

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10. REFERENCES

Anonymous (2004) If sports supplements and ergogenic aids work, they're probably dangerous and/or banned. *Drugs and Therapy Perspectives* 20:22-26.

Abelson, J. (2005) Creatine supplementation: the safety question. *Nutrition Noteworthy* 7:1-5.

Albrecht, E. W., Van Goor, H., Smit-van Oosten, A. and Stegeman, C. A. (2003) Long-term dietary *L*-arginine supplementation attenuates proteinuria and focal glomerulosclerosis in experimental chronic renal transplant failure. *Nitric Oxide - Biology and Chemistry* 8:53-58.

Aoki, M. S., Lima, W. P., Miyabara, E. H., Gouveia, C. H. A. and Moriscot, A. S. (2004) Deleterios effects of immobilization upon rat skeletal muscle: role of creatine supplementation. *Clinical Nutrition* 23:1176-1183.

Archer, M. C. (1999) Use of oral creatine to enhance athletic performance and its potential side effects. *Clinical Journal of Sport Medicine: Official Journal of the Canadian Academy of Sport Medicine* 9:119.

Archer, M. C. (2004a) Creatine: a safety concern. *Toxicology Letters* 152:275.

Archer, M. C. (2004b) The use of creatine to enhance athletic performance. *Nutrition* 20:841.

Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., DiMenna, F. J., Wilkerson, D. P., Tarr, J., Benjamin, N. and Jones, A. M. (2009) Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *Journal of Applied Physiology* 107:1144-1155.

Balimandawa, M., de Meester, C. and Léonard, A. (1993) *In vitro* study of clastogenic properties of nitrites and nitrates. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales* 187:744-750 (as cited in EFSA, 2008).

Bassit, R. A., Curi, R. and Costa Rosa, L. F. (2008) Creatine supplementation reduces plasma levels of pro-inflammatory cytokines and PGE_2 after a half-ironman competition. *Amino Acids* 35:425-431.

Berneburg, M., Gremmel, T., Kürten, V., Schroeder, P., Hertel, I., Von Mikecz, A., Wild, S., Chen, M., Declercq, L., Matsui, M., Ruzicka, T. and Krutmann, J. (2005) Creatine supplementation normalizes mutagenesis of mitochondrial DNA as well as functional consequences. *Journal of Investigative Dermatology* 125:213-220.

Bryan, N. S., Calvert, J. W., Elrod, J. W., Gundewar, S., Sang, Y. J. and Lefer, D. J. (2007) Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America* 104:19144-19149.

Brzezińska, Z., Nazar, K., Kaciuba-UśCiŁko, H., Falęcka-Wieczorek, I. and Wójcik-Ziółkowska, E. (1998) Effect of a short-term dietary creatine supplementation on high-energy phosphates in the rat myocardium. *Journal of Physiology and Pharmacology* 49:591-595.

CEC (2004) Working document for draft commission directive on foods intended to meet the expenditure of intense muscular effort, especially for sports people. Commission of the European Communities (CEC). Report Number: SANCO D4/HL/mm/D440182 p. 1-16.

Chyka, P. A. (2003) Health risks of selected performance-enhancing drugs. *Journal of Pharmacy Practice* 16:37-44.

Derave, W., Vanden Eede, E., Hespel, P., Carmella, S. G. and Hecht, S. S. (2006) Oral creatine supplementation in humans does not elevate urinary excretion of the carcinogen N-nitrososarcosine. *Nutrition* 22:332-333.

Edmunds, J. W., Jayapalan, S., DiMarco, N. M., Saboorian, M. H. and Aukema, H. M. (2001) Creatine supplementation increases renal disease progression in Han:SPRD-cy rats. *American Journal of Kidney Diseases* 37:73-78.

EFSA. (2004) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission related to creatine monohydrate for use in foods for particular nutritional uses. European Food Safety Authority (EFSA). *The EFSA Journal* 36:1-6.

EFSA. (2008) Nitrates in vegetables. Scientific opinion of the panel on contaminants in the food chain. European Food Safety Authority (EFSA). *The EFSA Journal* 689:1-79.

Eichholzer, M. and Gutzwiller, F. (1998) Dietary nitrates, nitrites, and *N*-nitroso compounds and cancer risk: a review of the epidemiologic evidence. *Nutrition Reviews* 56:95-105.

EPA (1991) National Primary Drinking Water Regulations; Synthetic Organic Chemicals and Inorganic Chemicals; Monitoring for Unregulated Contaminants; National Primary Drinking water Regulation Implementation; National Secondary Drinking Water Regulations; Final Rule. Federal Register. Vol. 56, No. 20, p.3526. January 30, 1991. United States Environmental Protection Agency (EPA).

EPA (2009) Consideration of other regulatory revisions in support of the second six-year review of the National Primary Drinking Water Regulations. United States Environmental Protection Agency (EPA). Office of Water (4607M). Report Number: EPA 815-B-09-008

EPA (2010) Revised Public Notification Handbook. 2nd Revision of document. United States Environmental Protection Agency (EPA). Office of Water (4606). Report Number: EPA 816-R-09-013

FAO/WHO (2003a) Nitrate (and potential endogenous formation of N-nitroso compounds). WHO Food Additive series 50. Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO).

FAO/WHO (2003b) Nitrite (and potential endogenous formation of *N*-nitroso compounds). WHO Food Additive series 50. Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO).

Farquhar, W. B. and Zambraski, E. J. (2002) Effects of creatine use on the athlete's kidney. *Current Sports Medicine Reports* 1:103-106.

Felton, J. S. and Knize, M. G. (2006) A meat and potato war: implications for cancer etiology. *Carcinogenesis* 27:2367-2370.

Fernandez, M. M. F. and Hosey, R. G. (2009) Performance-enhancing drugs snare nonathletes, too. *Journal of Family Practice* 58:16-23.

Francaux, M. and Poortmans, J. R. (2006) Side effects of creatine supplementation in athletes. *International Journal of Sports Physiology and Performance* 1:311-323.

Galbraith, R. A., Furukawa, M. and Li, M. (2006) Possible role of creatine concentrations in the brain in regulating appetite and weight. *Brain Research* 1101:85-91.

Gallo, M., Gordon, T., Syrotuik, D., Shu, Y., Tyreman, N., MacLean, I., Kenwell, Z. and Putman, C. T. (2006) Effects of long-term creatine feeding and running on isometric functional measures and myosin heavy chain content of rat skeletal muscles. *Pflugers Archiv European Journal of Physiology* 452:744-755.

Gallo, M., MacLean, I., Tyreman, N., Martins, K. J. B., Syrotuik, D., Gordon, T. and Putman, C. T. (2008) Adaptive responses to creatine loading and exercise in fast-twitch rat skeletal muscle. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 294:R1319-R1328.

George, T. W., Niwat, C., Waroonphan, S., Gordon, M. H. and Lovegrove, J. A. (2009) Effects of chronic and acute consumption of fruit- and vegetable-puree- based drinks on vasodilation, risk factors for CVD and the response as a result of the eNOS G298T polymorphism. *Proceedings of the Nutrition Society* 68:148-161.

Ghosh, M., Talukdar, D., Ghosh, S., Bhattacharyya, N., Ray, M. and Ray, S. (2006) *In vivo* assessment of toxicity and pharmacokinetics of methylglyoxal: augmentation of the curative effect of methylglyoxal on cancer-bearing mice by ascorbic acid and creatine. *Toxicology and Applied Pharmacology* 212:45-58.

Gualano, B., Ferreira, D. C., Sapienza, M. T., Seguro, A. C. and Lancha Jr., A. H. (2010) Effect of short-term high-dose creatine supplementation on measured GFR in a young man with a single kidney. *American Journal of Kidney Diseases* 55: e7-e9.

Guidi, C., Potenza, L., Sestili, P., Martinelli, C., Guescini, M., Stocchi, L., Zeppa, S., Polidori, E., Annibalini, G. and Stocchi, V. (2008) Differential effect of creatine on oxidatively-injured mitochondrial and nuclear DNA. *Biochimica et Biophysica Acta - General Subjects* 1780:16-26.

Harris, R. C., Soderlund, K. and Hultman, E. (1992) Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clinical Science* 83:367-374.

Hillbrand, M., Spitz, R. T., Foster, H. G., Krystal, J. H. and Young, J. L. (1998) Creatine kinase elevations and aggressive behavior in hospitalized forensic patients. *Psychiatric Quarterly* 69:69-82.

Holland-Hall, C. (2007) Performance-enhancing substances: is your adolescent patient using? *Pediatric Clinics of North America* 54:651-662.

Hord, N. G., Tang, Y. and Bryan, N. S. (2009) Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *American Journal of Clinical Nutrition* 90:1-10.

Horn, M., Frantz, S., Remkes, H., Laser, A., Urban, B., Mettenleiter, A., Schnackerz, K. and Neubauer, S. (1998) Effects of chronic dietary creatine feeding on cardiac energy metabolism and on creatine content in heart, skeletal muscle, brain, liver and kidney. *Journal of Molecular and Cellular Cardiology* 30:277-284.

Horn, M., Remkes, H., Dienesch, C., Hu, K., Ertl, G. and Neubauer, S. (1999) Chronic high-dose creatine feeding does not attenuate left ventricular remodeling in rat hearts post-myocardial infarction. *Cardiovascular Research* 43:117-124.

HSDB. (2010) Creatine. Hazardous Substance Data Base. In <<http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~CJRRaa:1 (site visited December 1, 2010)>>.

Hultman, E., Söderlund, K., Timmons, J. A., Cederblad, G. and Greenhaff, P. L. (1996) Muscle creatine loading in men. *Journal of Applied Physiology* 81:232-237.

IDACE (2003) Creatine monohydrate (Dossier) 22 January 2003. Submitted to the European Commission by Association des Industries des Aliments Dietetiques de l'Union Europeenne (IDACE). Paris, France (as cited in EFSA, 2004).

Ide, T., Chu, K., Aaronson, S. A. and Lee, S. W. (2010) GAMT joins the p53 network: branching into metabolism. *Cell Cycle* 9:1706-1710.

IOM. (2008) *Use of Dietary Supplements by Military Personnel.* Institute of Medicine (IOM) of the National Academies. In (M. R. C. Greenwood and M. OriaEds.) The National Academies Press, Washington DC. p. 1-457.

Ipsiroglu, O. S., Stromberger, C., Ilas, J., Höger, H., Mühl, A. and Stöckler-Ipsiroglu, S. (2001) Changes of tissue creatine concentrations upon oral supplementation of creatine-monohydrate in various animal species. *Life Sciences* 69:1805-1815.

Ju, J. S., Smith, J. L., Oppelt, P. J. and Fisher, J. S. (2005) Creatine feeding increases GLUT4 expression in rat skeletal muscle. *American Journal of Physiology- Endocrinology and Metabolism* 288:E347-E352.

Juhn, M. S. and Tarnopolsky, M. (1998) Oral creatine supplementation and athletic performance: a critical review. *Clinical Journal of Sport Medicine* 8:286-297.

Juhn, M. S. (2003) Popular sports supplements and ergogenic aids. *Sports Medicine* 33:921-939.

Kahn, T., Bosch, J., Levitt, M. F. and Goldstein, M. H. (1975) Effect of sodium nitrate loading on electrolyte transport by the renal tubule. *American Journal of Physiology* 229:746-753.

Kalinski, M. I. (2003) State-sponsored research on creatine supplements and blood doping in elite Soviet sport. *Perspectives in Biology and Medicine* 46:445-451.

Kapil, V., Milsom, A. B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F., Arghandawi, S., Pearl, V., Benjamin, N., Loukogeorgakis, S., MacAllister, R., Hobbs, A. J., Webb, A. J. and Ahluwalia, A. (2010) Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived no. *Hypertension* 56:274-281.

Koshy, K. M., Griswold, E., Schneeberger, E. E. and Thompson, R. W. (1999) Interstitial nephritis in a patient taking creatine [4] (multiple letters). *New England Journal of Medicine* 340:814-815.

Kosmicki, M. A. (2009) Long-term use of short- and long-acting nitrates in stable angina pectoris. *Current Clinical Pharmacology* 4:132-141.

Kreider, R. B., Melton, C., Rasmussen, C. J., Greenwood, M., Lancaster, S., Cantler, E. C., Milnor, P. and Almada, A. L. (2003) Long-term creatine supplementation does not significantly affect clinical markers of health in athletes. *Molecular and Cellular Biochemistry* 244:95-104.

Krul, C. A. M., Zeilmaker, M. J., Schothorst, R. C. and Havenaar, R. (2004) Intragastric formation and modulation of *N*-nitrosodimethylamine in a dynamic *in vitro* gastrointestinal model under human physiological conditions. *Food and Chemical Toxicology* 42:51-63.

Kuehl, K., Goldberg, L. and Elliot, D. (1998) Renal insufficiency after creatine supplementation in a college football athlete (abstract). *Medicine and Science in Sports and Exercise* 30:S235.

Larauche, M., Anton, P. M., Garcia-Villar, R., Theodorou, V., Frexinos, J., Buéno, L. and Fioramonti, J. (2003) Protective effect of dietary nitrate on experimental gastritis in rats. *British Journal of Nutrition* 89:777-786.

Larsen, F. J., Weitzberg, E., Lundberg, J. O. and Ekblom, B. (2007) Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiologica* 191:59-66.

Larsen, F. J., Weitzberg, E., Lundberg, J. O. and Ekblom, B. (2010) Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radical Biology and Medicine* 48:342-347.

Lehman, A. J. (1958) *Quarterly reports to the editor on topics of current interest - Nitrates and nitrites in meat products.* In: Association of Food and Drug Officials. 22:136-138.

Lijinsky, W., Greenblatt, M. and Kommineni, C. (1973) Brief communication: feeding studies of nitrilotriacetic acid and derivatives in rats. *Journal of the National Cancer Institute* 50:1061-1063.

Lowe, J. A., Murphy, M. and Nash, V. (1998) Changes in plasma and muscle creatine concentration after increases in supplementary dietary creatine in dogs. *Journal of Nutrition* 128:2691S-2693S.

Lundberg, J. O., Weitzberg, E., Cole, J. A. and Benjamin, N. (2004) Nitrate, bacteria and human health. *Nature Reviews Microbiology* 2:593-602.

Lundberg, J. O., Weitzberg, E. and Gladwin, M. T. (2008) The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery* 7:156-167.

Maekawa, A., Ogiu, T. and Onodera, H. (1982) Carcinogenicity studies of sodium nitrite and sodium nitrate in F-344 rats. *Food and Chemical Toxicology* 20:25-33.

Marsh, N. L., Iwaoka, W. T. and Mower, H. F. (1990) Formation of mutagens during the frying of Hawaiian fish: correlation with creatine and creatinine content. *Mutation Research* 242:181-186.

McGuire, M., Bradford, A. and MacDermott, M. (2002) Contractile properties of the diaphragm in creatine-fed rats. *Clinical and Experimental Pharmacology and Physiology* 29:782-783.

McLeish, M. J. and Kenyon, G. L. (2005) Relating structure to mechanism in creatine kinase. *Critical Reviews in Biochemistry and Molecular Biology* 40:1-20.

Mesa, J. L. M., Ruiz, J. R., Gonzálex-Gross, M. M., Gutiérrez Sáinz, Á. and Castillo Garzón, M. J. (2002) Oral creatine supplementation and skeletal muscle metabolism in physical exercise. *Sports Medicine* 32:903-944.

Milkowski, A., Garg, H. K., Coughlin, J. R. and Bryan, N. S. (2010) Nutritional epidemiology in the context of nitric oxide biology: a risk-benefit evaluation for dietary nitrite and nitrate. *Nitric Oxide - Biology and Chemistry* 22:110-119.

Moghadam, A. Z., Nazem, H., Karimi, I., Darani, I. A. and Hassanpour, H. (2008) Study of oral creatine monohydrate supplementation on growth performance and histopathological assessment in rats and chickens. *Journal of Biological Sciences* 8:436-440.

Moriya, A., Grant, J., Mowat, C., Williams, C., Carswell, A., Preston, T., Anderson, S., Iijima, K. and McColl, K. E. L. (2002) *In vitro* studies indicate that acid catalysed generation of *N*-nitrosocompounds from dietary nitrate will be maximal at the gastro-oesophageal junction and cardia. *Scandinavian Journal of Gastroenterology* 37:253-261.

Murkovic, M., Steinberger, D. and Pfannhauser, W. (1998) Antioxidant spices reduce the formation of heterocyclic amines in fried meat. *European Food Research and Technology* 207:477-480.

Nomura, A., Zhang, M., Sakamoto, T., Ishii, Y., Morishima, Y., Mochizuki, M., Kimura, T., Uchida, Y. and Sekizawa, K. (2003) Anti-inflammatory activity of creatine supplementation in endothelial cells *in vitro* . *British Journal of Pharmacology* 139:715-720.

NTP (2001) NTP technical report on the toxicology and carcinogenesis studies of sodium nitrite (CAS NO. 7632-00-0) in F344/N rats and B6C3F1 mice (drinking water studies). National Toxicology Program (NTP). Report Number: 495 p. 7-273.

Op 'T Eijnde, B., Richter, E. A., Henquin, J. -., Kiens, B. and Hespel, P. (2001) Effect of creatine supplementation on creatine and glycogen content in rat skeletal muscle. *Acta Physiologica Scandinavica* 171:169-176.

Osborne, N. N. (2008) Pathogenesis of ganglion "cell death" in glaucoma and neuroprotection: focus on ganglion cell axonal mitochondria. *Progress in Brain Research* 173:339-352.

Özkan, Ö., Duman, Ö., Haspolat, Ş., Özgentaş, H. E., Dikici, M. B., Gürer, I., Güngör, H. A. and Gökhan, A. G. (2005) Effect of systemic creatine monohydrate supplementation on denervated muscle during reinnervation: experimental study in the rat. *Journal of Reconstructive Microsurgery* 21:573-579.

Pandit, A., Mistry, P., Dib, P., Nikolaidis, A. and Dash, A. Intrinsic dissolution profiles of creatine nitrate, creatine monohydrate and buffered creatine (unpublished material).

PDR. (2001) Creatine. In *PDR for Nutritional Supplements. Physician's Desk Reference (PDR).* 1st Edition. Medical Economics Company, Montvale, NJ. p. 114-117.

Pecci, M. A. and Lombardo, J. A. (2000) Performance-enhancing supplements. *Physical Medicine and Rehabilitation Clinics of North America* 11:949-960.

Pfau, W., Rosenvold, K. and Young, J. F. (2006) Formation of mutagenic heterocyclic aromatic amines in fried pork from Duroc and Landrace pigs upon feed supplementation with creatine monohydrate. *Food and Chemical Toxicology* 44:2086-2091.

Pietraforte, D., Castelli, M., Metere, A., Scorza, G., Samoggia, P., Menditto, A. and Minetti, M. (2006) Salivary uric acid at the acidic pH of the stomach is the principal defense against nitrite-derived reactive species: sparing effects of chlorogenic acid and serum albumin. *Free Radical Biology and Medicine* 41:1753-1763.

Poortmans, J. R., Auquier, H., Renaut, V., Durussel, A., Saugy, M. and Brisson, G. R. (1997) Effect of short-term creatine supplementation on renal responses in men. *European Journal of Applied Physiology and Occupational Physiology* 76:566-567.

Poortmans, J. R. and Francaux, M. (1999) Long-term oral creatine supplementation does not impair renal function in healthy athletes. *Medicine and Science in Sports and Exercise* 31:1108-1110.

Poortmans, J. R. and Francaux, M. (2000) Adverse effects of creatine supplementation: fact or fiction? *Sports Medicine* 30:155-170.

Poortmans, J. R., Kumps, A., Duez, P., Fofonka, A., Carpentier, A. and Francaux, M. (2005) Effect of oral creatine supplementation on urinary methylamine, formaldehyde, and formate. *Medicine and Science in Sports and Exercise* 37:1717-1720.

Pritchard, N. R. and Kalra, P. A. (1998) Renal dysfunction accompanying oral creatine supplements. *Lancet* 351:1252-1253.

Radley, H. G., De Luca, A., Lynch, G. S. and Grounds, M. D. (2007) Duchenne muscular dystrophy: focus on pharmaceutical and nutritional interventions. *International Journal of Biochemistry and Cell Biology* 39:469-477.

Ralt, D. (2009) Does NO metabolism play a role in effects of vegetables in health? Nitric oxide formation via the reduction of nitrites and nitrates. *Medical Hypotheses* 73:794-796.

Reutersward, A. L., Skog, K. and Jagerstad, M. (1987a) Effects of creatine and creatinine content on the mutagenic activity of meat extracts, bouillons and gravies from different sources. *Food and Chemical Toxicology* 25:747-754.

Reutersward, A. L., Skog, K. and Jagerstad, M. (1987b) Mutagenicity of pan-fried bovine tissues in relation to their content of creatine, creatinine, monosaccharides and free amino acids. *Food and Chemical Toxicology* 25:755-762.

Ryu, H., Rosas, H. D., Hersch, S. M. and Ferrante, R. J. (2005) The therapeutic role of creatine in Huntington's disease. *Pharmacology and Therapeutics* 108:193-207.

Santamaria, P. (2006) Review: nitrate in vegetables: toxicity, content, intake and EC regulation. *Journal of the Science of Food and Agriculture* 86:10-17.

Santos, R. V. T., Batista Jr., M. L., Caperuto, É. C. and Costa Rosa, L. F. B. P. (2007) Chronic supplementation of creatine and vitamins C and E increases survival and improves biochemical parameters after doxorubicin treatment in rats. *Clinical and Experimental Pharmacology and Physiology* 34:1294-1299.

Saul, R. L., Kabir, S. H. and Cohen, Z. (1981) Reevaluation of nitrate and nitrite levels in the human intestine. *Cancer Research* 41:2280-2283.

SCF (2000) Opinion of the Scientific Committee on Food (SCF) on safety aspects of creatine supplementation. Report Number: SCF/CS/NUT/SPORT/9 Final p. 1-6.

SCF (2001) Report of the Scientific Committee on Food (SCF) on composition and specification of food intended to meet the expenditure of intense muscular effort, especially for sportsmen. Report Number: SCF/CS/NUT/SPORT/5 Final (corrected)

Schroeder, C., Potteiger, J., Randall, J., Jacobsen, D., Magee, L., Benedict, S. and Hulver, M. (2001) The effects of creatine dietary supplementation on anterior compartment pressure in the lower leg during rest and following exercise. *Clinical Journal of Sport Medicine* 11:87-95.

Schulze, A. (2003) Creatine deficiency syndromes. *Molecular and Cellular Biochemistry* 244:143-150.

Schut, H. A. J. and Snyderwine, E. G. (1999) DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis* 20:353-368.

Shao, A. and Hathcock, J. N. (2006) Risk assessment for creatine monohydrate. *Regulatory Toxicology and Pharmacology* 45:242-251.

Shephard, S. E., Schlatter, C. and Lutz, W. K. (1987) Assessment of the risk of formation of carcinogenic *N*-nitroso compounds from dietary precursors in the stomach. *Food and Chemical Toxicology* 25:91-108.

Sobko, T., Huang, L., Midtvedt, T., Norin, E., Gustafsson, L. E., Norman, M., Jansson, E. Å. and Lundberg, J. O. (2006) Generation of NO by probiotic bacteria in the gastrointestinal tract. *Free Radical Biology and Medicine* 41:985-991.

Sobko, T., Marcus, C., Govoni, M. and Kamiya, S. (2010) Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. *Nitric Oxide - Biology and Chemistry* 22:136-140.

Speijers, G. J. A., Van Went, G. F., Van Apeldoorn, M. E., Montizaan, G. K., Janus, J. A., Canton, J. H., Van Gestel, G. A. M., Van der Heijden, C. A., Heijna-Merkus, E., Knapp, A. G. A. C., Luttick, R. and Zwart, D. (1987) Integrated Criteria document Nitrate Effects. Appendix to RIVM. Report Number: 758473007. Bilthoven (cited in EFSA, 2008)

Stead, L. M., Au, K. P., Jacobs, R. L., Brosnan, M. E. and Brosnan, J. T. (2001) Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. *American Journal of Physiology - Endocrinology and Metabolism* 281:

Sugekawa, K. and Matsumoto, T. (1975) Nitrate and nitrite contents in cow and human milk. *Eyio To Shokuryo* 28:389-393. (abstract in Chemical Abstracts 84, 102816) (cited in EFSA, 2008).

Taes, Y. E. C., Delanghe, J. R., Wuyts, B., van de Voorde, J. and Lameire, N. H. (2003) Creatine supplementation does not affect kidney function in an animal model with pre-existing renal failure. *Nephrology Dialysis Transplantation* 18:258-264.

Taes, Y. E. C. and De Vriese, A. S. (2005) Analytical and biochemical aspects associated with supraphysiological creatine intake. *Clinica Chimica Acta* 351:217-219.

Takahama, U., Hirota, S. and Takayuki, O. (2008) Detection of nitric oxide and its derivatives in human mixed saliva and acidified saliva. *Methods in Enzymology* 440:381-396.

Thermo-Life International. (2010) Stability data. (Personal Communication)

Thomasos, C. (2007) Assessment of air force dietary supplement usage by major commands. Institute of Medicne Committee on Dietary Supplemenst Use by Military Personnel meeting, Washington, DC, February 13. (as cited in IOM, 2008).

Tsezou, A., Kitsiou-Tzeli, S., Galla, A., Gourgiotis, D., Papageorgiou, J., Mitrou, S., Molybdas, P. A. and Sinaniotis, C. (1996) High nitrate content in drinking water: cytogenetic effects in exposed children. *Archives of Environmental Health* 51:458-461.

Vikse, R. and Joner, P. E. (1993) Mutagenicity, creatine and nutrient contents of pan fried meat from various animal species. *Acta Veterinaria Scandinavica* 34:363-370.

Vithayathil, A. J., Strasik, M. and Strasik, L. (1983) Heat-induced mutagen formation from creatine and fat-soluble constituents of food. *Mutation Research* 121:167-170.

Walker, R. (1990) Nitrates, nitrites and *N*-nitrosocompounds: a review of the occurrence in food and diet and the toxicological implications. *Food Additives and Contaminants* 7:717-768.

Williams, M.H. and Branch, J.D. (1998). Creatine supplementation and exercise performance: an update. *Journal of American College of Nutrition.* 17:216-234.

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Marc Rhoads, J., Carey Satterfield, M., Smith, S. B., Spencer, T. E. and Yin, Y. (2009) Arginine metabolism and nutrition in growth, health and disease. *Amino acids* 37:153-168.

Wyss, M. (2004) Writing about creatine: is it worth the risk? *Toxicology Letters* 152:273-274.

Yoshida, D. and Okamoto, H. (1980) Formation of mutagens by heating creatine and glucose. *Biochemical and Biophysical Research Communications* 96:844-847.

11. APPENDIX 1. Double-blind, randomized, controlled human clinical trials cited in Shao and Hathcock, 2006.

Arciero, P. J., Hannibal III, N. S., Nindl, B. C., Gentile, C. L., Hamed, J. and Vukovich, M. D. (2001) Comparison of creatine ingestion and resistance training on energy expenditure and limb blood flow. *Metabolism: Clinical and Experimental* 50:1429-1434.

Chrusch, M. J., Chilibeck, P. D., Chad, K. E., Davison, K. S. and Burke, D. G. (2001) Creatine supplementation combined with resistance training in older men. *Medicine and Science in Sports and Exercise* 33:2111-2117.

Derave, W., Eijnde, B. O., Verbessem, P., Ramaekers, M., Van Leemputte, M., Richter, E. A. and Hespel, P. (2003) Combined creatine and protein supplementation in conjunction with resistance training promotes muscle GLUT-4 content and glucose tolerance in humans. *Journal of Applied Physiology* 94:1910-1916.

Derave, W., Marescau, B., Vanden Eede, E., Eijnde, B. O., De Deyn, P. P. and Hespel, P. (2004) Plasma guanidino compounds are altered by oral creatine supplementation in healthy humans. *Journal of Applied Physiology* 97:852-857.

Deuster, P. A., Bennett, T., Bathalon, G., Armstrong III, D., Martin, B., Coll, R., Beck, R., Barkdull, T. and O'Brien, K. (2001) Effect of creatine on performance of militarily relevant tasks and soldier health. *Military Medicine* 166:996-1002.

Eijnde, B. O., Van Leemputte, M., Goris, M., Labarque, V., Taes, Y., Verbessem, P., Vanhees, L., Ramaekers, M., Vanden Eynde, B., Van Schuylenbergh, R., Dom, R., Richter, E. A. and Hespel, P. (2003) Effects of creatine supplementation and exercise training on fitness in men 55-75 yr old. *Journal of Applied Physiology* 95:818-828.

Escolar, D. M., Buyse, G., Henricson, E., Leshner, R., Florence, J., Mayhew, J., Tesi-Rocha, C., Gorni, K., Pasquali, L., Patel, K. M., McCarter, R., Huang, J., Mayhew, T., Bertorini, T., Carlo, J., Connolly, A. M., Clemens, P. R., Goemans, N., Iannaccone, S. T., Igarashi, M., Nevo, Y., Pestronk, A., Subramony, S. H., Vedanarayanan, V. V. and Wessel, H. (2005) CINRG randomized controlled trial of creatine and glutamine in Duchenne muscular dystrophy. *Annals of Neurology* 58:151-155.

Groeneveld, G. J., Beijer, C., Veldink, J. H., Kalmijn, S., Wokke, J. H. J. and Van Den Berg, L. H. (2005) Few adverse effects of long-term creatine supplementation in a placebo-controlled trial. *International Journal of Sports Medicine* 26:307-313.

Kilduff, L. P., Pitsiladis, Y. P., Tasker, L., Attwood, J., Hyslop, P., Dailly, A., Dickson, I. and Grant, S. (2003) Effects of creatine on body composition and strength gains after 4 weeks of resistance training in previously nonresistance-trained humans. *International Journal of Sport Nutrition and Exercise Metabolism* 13:504-520.

Kreider, R. B., Ferreira, M., Wilson, M., Grindstaff, P., Plisk, S., Reinardy, J., Cantler, E. and Almada, A. L. (1998) Effects of creatine supplementation on body composition, strength, and sprint performance. *Medicine and Science in Sports and Exercise* 30:73-82.

Louis, A., Lebacq, J., Poortmans, J. R., Belpaire-Dethiou, M., Devogelaer, J., Van Hecke, P., Goubel, F. and Francaux, M. (2003) Beneficial effects of creatine supplementation in dystrophic patients. *Muscle and Nerve* 27:604-610.

Op 'T Eijnde, B., Urso, B., Richter, E. A., Greenhaff, P. L. and Hespel, P. (2001) Effect of oral creatine supplementation on human muscle GLUT4 protein content after immobilization. *Diabetes* 50:18-23.

Powers, M. E., Arnold, B. L., Weltman, A. L., Perrin, D. H., Mistry, D., Kahler, D. M., Kraemer, W. and Volek, J. (2003) Creatine supplementation increases total body water without altering fluid distribution. *Journal of Athletic Training* 38:44-50.

Robinson, T. M., Sewell, D. A., Casey, A., Steenge, G. and Greenhaff, P. L. (2000) Dietary creatine supplementation does not affect some haematological indices, or indices of muscle damage and hepatic and renal function. *British Journal of Sports Medicine* 34:284-288.

Stevenson, S. W. and Dudley, G. A. (2001) Dietary creatine supplementation and muscular adaptation to resistive overload. *Medicine and Science in Sports and Exercise* 33:1304-1310.

Tarnopolsky, M., Mahoney, D., Thompson, T., Naylor, H. and Doherty, T. J. (2004a) Creatine monohydrate supplementation does not increase muscle strength, lean body mass, or muscle phosphocreatine in patients with myotonic dystrophy type 1. *Muscle and Nerve* 29:51-58.

Tarnopolsky, M. A., Mahoney, D. J., Vajsar, J., Rodriguez, C., Doherty, T. J., Roy, B. D. and Biggar, D. (2004b) Creatine monohydrate enhances strength and body composition in Duchenne muscular dystrophy. *Neurology* 62:1771-1777.

Van Loon, L. J. C., Oosterlaar, A. M., Hartgens, F., Hesselink, M. K. C., Snow, R. J. and Wagenmakers, A. J. M. (2003) Effects of creatine loading and prolonged creatine supplementation on body composition, fuel selection, sprint and endurance performance in humans. *Clinical Science* 104:153-162.

Verbessem, P., Lemiere, J., Eijnde, B. O., Swinnen, S., Vanhees, L., Van Leemputte, M., Hespel, P. and Dom, R. (2003) Creatine supplementation in Huntington's disease: a placebo-controlled pilot trial. *Neurology* 61:925-930.

Walter, M. C., Lochmüller, H., Reilich, P., Klopstock, T., Huber, R., Hartard, M., Hennig, M., Pongratz, D. and Müller-Felber, W. (2000) Creatine monohydrate in muscular dystrophies: a double-blind, placebo- controlled clinical study. *Neurology* 54:1848-1850.

Watsford, M. L., Murphy, A. J., Spinks, W. L. and Walshe, A. D. (2003) Creatine supplementation and its effect on musculotendinous stiffness and performance. *Journal of Strength and Conditioning Research* 17:26-33.

Wilder, N., Deivert, R. G., Hagerman, F. and Gilders, R. (2001) The effects of low-dose creatine supplementation versus creatine loading in collegiate football players. *Journal of Athletic Training* 36:124-129.